MUTATIONAL AND BIOCHEMICAL ANALYSIS OF THE CELL CYCLE IN *CHLAMYDOMONAS REINHARDTII*

BY

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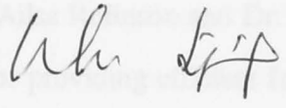
THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
OF THE AUSTRALIAN NATIONAL UNIVERSITY

DECEMBER, 1993

STATEMENT

All the research reported in this thesis is original
and my own, except where due acknowledgment is made,
and has not submitted for any other degree.



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ACKNOWLEDGMENTS

I am grateful to my supervisor Dr. Pete John and to Plant Cell Biology Group who provided me the chance to come to Australia to complete this study. Pete's advice, help, encouragement, and patience throughout the course of the study are greatly appreciated. I would also like to thank my adviser Prof. Brian Gunning for his encouragement and support during my study.

I wish to acknowledge all those who have helped me in the past four years. In particular, I thank Mr. Frank Sek and Dr. Suchirat Sakuanrungsirikul who helped me from the very beginning; Dr. Charles Hocart, Chongmei Dong, Kongnan Zhao and Kerong Zhang for their help in numerous aspects; Dr. Ludger Diederich for his advice on experimental techniques and help in preparation of figures; Dr. Ute Schuppler for kind advice in experiments; Prof. Peter Hepler for microinjection; Dr. John Harper for providing useful references; Ailsa Rolinson and Dr. Sandra Jackson for help in confocal microscopy; Pat Wilkinson for providing efficient facilities. I have also enjoyed sharing the laboratory with Kim Badelt, Penny Webb and Juliet Morris.

I especially thank Qiao Lin, Yanhong Wu, Shigang He, and Liqun Wang for their friendship, which has made my stay in Canberra more exciting and meaningful. I am also indebted to Prof. Hongjun Hu and Prof. Annette Coleman; their friendship, care and provision of opportunities for increasing my knowledge are greatly appreciated.

I owe a lot to my parents whose unreserved love, encouragement, understanding and support have been a great part of the energy that has driven me to complete this study.

Sincere thanks go to my husband Zhiping Yan who has shared all my happiness, excitement and frustrating throughout all of these years and to our daughter Xiaorui Yan for her tolerance of my absence and for all the happiness that she has brought to me.

Finally, I would like to acknowledge the Plant Cell Biology Group and the Academia Sinica for the financial support that has made this study possible.

ABSTRACT

The unicellular plant *Chlamydomonas* was used as a model system to study the plant cell division cycle, by investigation of both wild type and conditional (temperature sensitive) cell division cycle mutants.

Synchronous wild type cells were used to investigate normal cell structure and biochemical changes during the cell cycle. Regression of flagella was initiated after cells attained commitment to division and just before initiation of DNA synthesis. This timing is discussed in relation to mutant phenotypes. Using an antibody against the conserved PSTAIR sequence of $p34^{cdc2}$, four bands of $p34^{cdc2}$-like protein with apparent molecular weights of 32, 34, 36 and 38 kDa were identified. Their identity as possible phosphorylation forms of $p34^{cdc2}$ protein or variants of $p34^{cdc2}$ is discussed. Both the level of $p34^{cdc2}$-like histone H1 kinase activity purified by affinity for $p13^{suc1}$ and the level of the $p34^{cdc2}$-like protein detected by anti PSTAIR antibody peaked to 8 fold and 3-4 fold above the initial G1 level respectively when mitotic activity was maximal.

Five cdc ts mutants were characterised by analysis of their nuclear DNA contents (by PI staining and confocal laser scanning) and cytoskeletal structures (by indirect immunofluorescence microscopy). The mutants were able to undergo cell division normally at 21°C, but when they were cultured at the restrictive temperature of 33°C they arrested respectively in G1, G2, mitosis and cytokinesis phases.

The G1-arresting mutant designated *cdG1-1* was able to attain commitment to divide and to withdraw flagella at the restrictive temperature, but not able either to replicate nuclear DNA, depolymerise interphase cortical microtubules or initiate progress to cytokinesis. The mutation identified a function that is necessary for completion of G1 phase and initiation of nuclear DNA synthesis.

Two independently isolated G2-arresting mutants, *cdM-1* and *cdM-2*, were found to arrest with very similar terminal phenotypes except that they attained different arrested cell sizes and the partial activation of $p34^{cdc2}$-like kinase was more extensive in arrested cells of *cdM-1*. The two mutants arrested with withdrawn flagella and doubled nuclear

DNA content but failed to depolymerise their cortical microtubules and were unable to initiate mitosis. Biochemical analysis demonstrated that the *cdM-1* mutation caused cells arrested with partially activated $p34^{cdc2}$-like histone H1 kinase activity and $p34^{cdc2}$-like protein level indicative of preprophase.

Levels of a protein of 13 kDa that crossreacted with antibody against the 13 kDa protein $p13^{suc1}$, which is essential for mitotic anaphase in fission yeast, remained at a constant proportion of total protein throughout the cell cycle of mutants *cdM-1* and *met-1* at both permissive and restrictive temperatures and is considered not to be the cause of arrest by the cdc mutations studied here.

A mitotic-arresting mutant, *met-1*, arrested with fully formed spindles and condensed chromosomes of 2C DNA content that were aligned at a metaphase plate. Partial initiation of cytokinesis was observed in many arrested cells, which supported the early conclusion (Harper and John, 1986) that progress to cytokinesis occurred in a sequence of events independent of the DNA-division sequence. Levels of both $p34^{cdc2}$-like histone H1 kinase activity and of the $p34^{cdc2}$-like protein remained high in arrested cells of *met-1*. Failure to inactivate the $p34^{cdc2}$ protein kinase correlated with persistence of cyclin B like protein. The mutation therefore identified a gene function that is necessary for the metaphase-anaphase transition. The highly active $p34^{cdc2}$ was affinity purified from the *met-1* arrested cells and shown to have activity by the functional test of microinjection into stamen hair cells of the higher plant *Tradescantia virginiana*. When injected into early prophase cells it induced rapid progress to metaphase followed by normal completion of nuclear division, indicating that arrest of *met-1* was not caused by an altered MPF catalytic function or presence of an inherently nondegradable cyclin.

A cytokinesis-arresting mutant, *cdCK-1*, could undergo up to three normal rounds of nuclear division to form multiple daughter nuclei containing the normal G1 nuclear DNA content, but the arrested cells were unable to initiate cytoplasmic cleavage or form a phycoplast between the nuclei although some loosely aligned microtubules that might be aberrant phycoplast could be observed in the region between the newly formed daughter nuclei. The *cdCK-1* mutation therefore identified a gene function that is necessary for completion of cytokinesis.

The microtubule depolymerising drug oryzalin arrested wild type cells in prophase with multinucleate arrest phenotype but did not block the mitotic oscillation of the $p34^{cdc2}$ kinase activity. After cells were released from 9 h oryzalin treatment, they could complete the current cell cycle but arrested cells did not attempt cytokinesis. The significance of this is discussed in relation to checkpoint control and the observation of attempted cytokinesis in arrested *met-1* cells.

The time period during the cell cycle in which the proteins modified by the cdc mutation performed their function was investigated by temperature shifting experiments. A catastrophe point was identified as the mean time at which the population lost its capacity to complete cell division on transfer back to permissive temperature and it represents the time at which lack of the mutated protein causes irretrievable abnormal development. The execution point of the mutants, at which time sufficient of the function of the mutated protein had been completed for division to occur, was identified as the mean time at which cells were able to complete the cell division on transfer to restrictive temperature. In each mutant the timing of catastrophe point preceded the execution point. The possibility is discussed that between the catastrophe and execution points the proteins identified by mutation normally made their contribution to the cell cycle. The catastrophe point of the *met-1* mutant was the latest of the studied mutants, which may correlate with a function in mid mitosis. An early catastrophe point in *cdM-1* and *cdM-2* prior to S phase may indicate that early preparations for mitosis begin long before the structural changes are seen.

Genetic analysis of the mutants included: (1) repeated backcrossing to obtain each mutant as a single gene mutation in a wild type background; (2) the metaphase arresting mutant *met-1* was mapped into the genetic linkage group XIV, located on the same side of the centromere as the *ery2* marker with a mapping distance of 32.4 cM from the marker. The linkage and the locus were confirmed by three point crosses; (3) two suppressor cell lines were isolated by secondary mutation of the *met-1* mutant cells, suggesting that the *met-1* gene product may interact with other proteins in regulation of the metaphase-anaphase transition.

ABBREVIATIONS

Amp	ampicillin
ATP	adenosine -5'-triphosphate
BSA	bovine serum albumin
BCIP	5-bromo-4-chloro-3-indolylphosphate p-toluidine
cAMP	cyclic adenosine-3',5'-monophosphate
cdc	cell division cycle
cdc2	cell division cycle gene identified in fission yeast by mutation and arbitrarily numbered 2. The equivalent gene in budding yeast is designated CDC28 or *CDC28*; mutant forms of these genes are designated *cdc2* or *cdc2*$^{-}$ and cdc28 respectively; following the conventions of the two organisms. In this thesis where the generic type of genes is referred to in other eukaryotes (rather than the specific gene in yeast), the simpler designation cdc2 is used. Throughout, the protein products are referred to as p34^{cdc2} protein to indicate its 34 kDa size, or as cdc2 protein or CDC28 protein. The same conventions, with respect to use of upper and lower case and italics, are followed with other cell cycle genes (except in *Aspergillus*, *nimA* refers to the gene and NIMA refers to the protein product).
cdk	cyclin dependent kinase
cM	centi Morgan
CNBr	cyanogen bromide
cpm	counts per minute
DAPI	4', 6 -diamiedino-2-phenylindole
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol

EDTA	ethylenediamine N, N, N', N'-tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N, N, N', N'-tetraacetic acid
EMS	ethyl methane sulphonate
FITC	fluorescein isothiocyanate
fl	femto litre
G1	gap1, period after mitosis, before DNA synthesis
G2	gap2, period after DNA synthesis, before mitosis
h	hour
HDW	high detergent washing buffer, defined in methods section
HeLa	line of human neoplastic (cervical) epithelial cells
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
PI	propidium iodide
kb	kilobase
kDa	kilodalton
l	litre
LB medium	Luria-Bertani medium
μ	micro
m	milli
min	minute
M	molarity
MPF	maturation or mitosis promoting factor
MTSB	microtubule stabilising buffer, defined in methods section
NBT	nitroblue tetrazolium chloride
NDE	nondetergent extraction buffer, defined in methods section
NP-40	NONIDET P-40, nonionic detergent
NPD	nonparental ditype
OD	optical density
PBS	phosphate buffered saline
PD	parental ditype

PIPES	piperazine-N, N'-bis(2-ethane-sulphonic acid)
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
RF	recombination frequency
rpm	revolutions per minute
sec	second
SDS	sodium dodecyl sulphate
T	tetratype
Tris	tris(hydroxymethyl)-aminomethane
Tween 20	polyxythylenesorbitan monolaurate, nonionic detergent
ts	temperature sensitive
UV	ultra violet

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SECTION 1. GENERAL INTRODUCTION

1.1. The cell cycle

Fundamental elements of the cell division cycle appear to be similar in all eukaryotes. The cycle is traditionally divided into four phases: G1 (gap1), S (DNA synthesis), G2 (gap2) and M (mitosis) (Howard and Pelc, 1953) and currently two major regulatory transitions at G1/S and G2/M are recognised. During S phase, cells replicate their nuclear DNA exactly once, so allowing the later separation of two identical sets of chromosomes through the action of the mitotic spindle in mitosis. The time interval between mitosis and DNA synthesis in the subsequent cell cycle is named G1 phase and can occupy many hours while new daughter cells grow in size. Few special cell cycle events are seen during this period (Watson et al., 1992) although synthesis of G1 cyclins may be necessary especially in late G1, which is terminated by the START division events that lead rapidly to S phase (Reed, 1991). After completion of DNA replication cells are in G2 phase may include changes leading to mitosis, especially in higher plant cells where the preprophase band of cortical microtubules becomes progressively more tightly focused through G2 phase (Gunning and Sammut, 1990).

During the past two decades, data from genetic, biochemical and molecular investigations in diverse organisms, especially the cloning of some key cell cycle control genes, has given new insight into understanding the control mechanisms of the cell cycle and an understanding that certain aspects are universal in all eukaryotes (Lee and Nurse, 1987; Moreno et al., 1989a; Nurse, 1990).

1.2. Study of cell cycle control using yeasts as model systems

Because of the possibility that all eukaryotes could have similar cell cycle controls, some scientists sensibly started to explore the cell cycle in simple experimental systems. Two of the most successfully used are *Saccharomyces cerevisiae* (budding yeast) and *Schizosaccharomyces pombe* (fission yeast). The small nuclear genome and well characterised genetic background of yeasts, complemented by well established

genetic and molecular techniques, makes them suitable for gene cloning and for manipulation with recombinant DNA. Since yeasts are unicellular, can be haploid and have a cell cycle that can be as short as 2 hours, they can be readily grown in large populations for both biochemical analysis of cell cycle gene products and for study of the effects of cell division cycle (cdc) mutants.

Rate of cell division in yeasts is coordinated with rate of growth. Progress through a key rate-limiting control point called START in late G1 of the cell cycle requires a minimum cell size and this requirement therefore adjusts division rate to growth rate (reviewed by Fantes and Nurse, 1981; John et al., 1993a). Cells that can pass through START will complete the current cell cycle without requirement for further growth. Equivalents of the irreversible control point have also been found in other organisms such as the Commitment Point in *Chlamydomonas* (Donnan et al., 1985; McAteer et al., 1985; John, 1987) and the Restriction Point in cultured mammalian cells (Pardee, 1989). The coordination of cell division with growth has also been detected in some cell types at a second regulatory point between G2 and mitosis. In fission yeast, initiation of mitosis is modulated by nutrition and is believed to require an adequate cell size (Nurse and Fantes, 1981). When growth is rapid, cells initiate mitosis at larger sizes and produce daughter cells that are big enough in their next cell cycle to execute START after a minimum time in G1. The two processes of growth and cell division when in balance allow a stable cell size and a defined cell morphology to be maintained.

It is difficult to study the control of the cell cycle without a means to upset its progress (Howell, 1974). A powerful way of interfering with the cell cycle has been the use of cell division cycle (cdc) mutants. For this purpose mutations that cause complete blockage of cell cycle progress under all conditions are not valuable because they are lethal. It is, however, possible to isolate conditional mutants that make altered proteins that are functional under one condition but nonfunctional under another. This conditional type of mutant has been widely used with increased temperature as the restrictive condition.

Isolation of temperature sensitive (ts) cell cycle mutants was first initiated in the budding yeast (*S. cerevisiae*) by Hartwell and his colleagues and resulted in a collection

of mutants that arrested in different stages of the cell cycle (Hartwell et al., 1970; Culotti and Hartwell, 1971; Hartwell, 1973; Hartwell et al., 1974). Up to now, approximately 60 different cdc genes have been cloned in *S. cerevisiae* by complementation of these mutants. Among these genes, *CDC28* was cloned by Reed (1980) and originally identified as the primary regulator that acts only at START. This is the key event in late G1 that commits cells to complete the current cell cycle in spite of any subsequent detection of mating pheromone or reduction in growth rate. The ts *CDC28* mutant was able to divide normally at 23°C. Cells that attained the critical minimum cell size then executed a START division event in late G1 and were able to bud and to initiate DNA synthesis followed by nuclear division and cytokinesis. When the temperature was shifted from 23°C to 37°C, the *CDC28* mutant was unable to perform START and arrested as large unbudded cells in late G1 without initiation of DNA synthesis. A smaller collection of ts cell cycle mutants was also isolated later in *S. pombe* by Nurse and his colleagues and a gene called cdc2 was cloned and found to function at both START and the G2/M transition (Nurse et al., 1976; Nurse,1981; Nurse and Bissett, 1981). Cloning and sequencing of the *cdc2* and *CDC28* genes revealed that they both encode proteins of 34 kDa that belong to the family of protein kinases. These two proteins shared 60% homology of amino acid sequence including the highly conserved PSTAIR sequence present in all cdc2 homologues (Lee and Nurse, 1987). A mutant of *CDC28* that had lost G2/M activity was later isolated (Piggott and Carter, 1982; Wittenberg and Reed, 1989; Surana et al., 1993). This mutant arrested entirely at G2/M, which clearly showed that the *CDC28* gene has a G2/M function. In a classic experiment, Beach Durkacz and Nurse (1982) showed that *CDC28* could complement a *cdc2* mutant in *S. pombe*; except that the cells appeared to be wee (small at mitosis) in phenotype. It was later also found that the *cdc2* gene with introns removed could complement a *CDC28* mutation in *S. cerevisiae* (Booher and Beach, 1986). Therefore, *cdc2* and *CDC28* genes are functionally interchangeable (Beach et al., 1982; Hindley and Phear, 1984; Reed and Wittenberg, 1990).

1.3. Conserved control of the cell cycle

The similarity between the products of the *cdc2* and *CDC28* genes in the two distantly related yeasts *S. pombe* and *S. cerevisiae* indicated the possibility that such a conserved gene product might also exist in other eukaryotic organisms. Investigation was first extended to human cells by Lee and Nurse (1987). By complementation of a temperature sensitive mutation of the *cdc2* gene in fission yeast with a human cDNA, they successfully cloned a human cdc2 gene, *cdc2Hs* (Hs for *Homo sapiens*). Comparison of the predicted amino acid sequences showed that the *cdc2Hs* product was 63% identical with $p34^{cdc2}$ of the fission yeast and 58% with $p34^{CDC28}$ of the budding yeast. When transferred into a fission yeast *cdc2* mutant, the human cdc2 gene was able to complement all known *cdc2*-encoded functions. At this time, Draetta and his colleagues also identified a 34 kDa protein kinase in human HeLa cells using monoclonal antibody against fission yeast $p34^{cdc2}$ (Draetta et al., 1987; Draetta and Beach, 1989). Proteolytic mapping, kinase activity of the immunoprecipitates and association of the human protein with the homologue of the *suc1* gene product of fission yeast, which is a binding protein of $p34^{cdc2}$, confirmed that the precipitated 34 kDa protein kinase in HeLa cells was a human homologue of the fission yeast *cdc2* gene product.

About the same time as *CDC28* and *cdc2* were discovered, cytological and biochemical analysis of *Xenopus laevis* oocytes identified an activity that was present in metaphase cytoplasm of maturing oocytes and unfertilised eggs that could cause immature oocytes to undergo oocyte maturation (Masui and Markert, 1971; Doree, 1990). This activity was described as maturation-promoting factor (MPF). When microinjected into immature oocytes that were naturally arrested in premeiotic G2, the MPF from metaphase arrested oocytes could promote meiotic progression (Masui and Markert, 1971; Smith and Ecker, 1971). The number of species of active molecule in this crude preparation was entirely unknown. Highly purified MPF was later found to consist of a complex of a 34 kDa serine/threonine protein kinase, identified as a *Xenopus* homologue of the *cdc2* gene product , $p34^{cdc2}$, and a 45 kDa substrate, identified as a *Xenopus* B-type cyclin. When the PSTAIR antibody (antibody against the 16 amino acid sequence

EGVPSTAIREISLLKE that is perfectly conserved in cdc2 proteins from yeasts to human, (Lee and Nurse, 1987)) was used on Western blots of the purified MPF preparations, it revealed the 34 kDa component of MPF co-migrating with authentic human $p34^{cdc2}$ (Gautier et al., 1988). Identification of $p34^{cdc2}$ as the kinase component of MPF, which is today recognised as the regulator of G2/M transition, further confirmed the universality of cell cycle control in diverse organisms.

To date, homologues of the *cdc2* gene or gene product have been found in a variety of eukaryotes including sea urchin (Meijer et al., 1989), starfish (Arion et al., 1988), *Aspergillus* (Osmani et al., 1991), *Drosophila* (Lehner and O'Farrell, 1990), clam (Draetta et al., 1989), *Chlamydomonas* (John et al., 1989), *Arabidopsis*, Oats (John et al., 1989, Hirayama et al., 1991; Ferreira et al., 1991), maize (Colasanti et al., 1991), alfalfa (Hirt et al., 1991; Hirt et al., 1993) and soybean (Miao et al., 1993). The key cell cycle regulating protein $p34^{cdc2}$ is present in all eukaryotes and the fundamental mechanism that controls mitotic initiation in all eukaryotes is probably universal (Nurse, 1990).

1.4. Control of $p34^{cdc2}$ protein kinase activity by phosphorylation

Investigation of the properties of the $p34^{cdc2}$ protein kinase at different stages of the cell cycle has been carried out to try to understand the role of $p34^{cdc2}$ at the G1/S and G2/M transitions.

The $p34^{cdc2}$ kinase contains a putative ATP-binding site close to the amino terminal and a 16 amino acid sequence EGVPSTAIRELLKE (PSTAIR) encoded by nucleotides 126-174 (in *S. pombe*) which is perfectly conserved in all known cdc2-like serine/threonine protein kinases. It has been found that the level of $p34^{cdc2}$ protein is relatively constant relative to other proteins throughout the cell cycle, but phosphorylation and catalytic activity of the protein kinase varies with cell cycle phase (Draetta and Beach, 1989). As a protein kinase, $p34^{cdc2}$ can transfer phosphate groups from ATP onto the hydroxyl groups of serine/threonine residues of some cell cycle

related proteins, of which histone H1 is a particularly favoured substrate *in vitro*. The $p34^{cdc2}$ protein itself can be phosphorylated or dephosphorylated on its serine/threonine and tyrosine residues by other protein kinases and phosphatases directly affecting its kinase activity.

There are four phosphorylation sites that are involved in regulating the activity of the $p34^{cdc2}$ protein kinase. These four sites have been identified by a combination of genetic methods and phosphopeptide mapping. The tyrosine residue at site 15 (Tyr15 or Y15), in the putative ATP-binding site of *S. pombe* $p34^{cdc2}$, when mutated to phenylalanine caused the cells to enter mitosis prematurely. Dephosphorylating this tyrosine in wild type $p34^{cdc2}$ isolated from G2 cells using human protein-tyrosine phosphatase activates histone H1 kinase activity. This indicates that dephosphorylation on Tyr15 alone is sufficient to activate the G2 form of *S. pombe* $p34^{cdc2}$ protein kinase that has previously been phosphorylated at Thr161 and complexed with cyclin B (Gould and Nurse, 1989; Clarke and Karsenti, 1991). Tyr15 has also been found to be a conserved phosphorylation site in vertebrate $p34^{cdc2}$. However, in addition to Tyr15 the adjacent threonine residue (Thr14) is also phosphorylated in higher eukaryotes, as first observed in synchronised G2 cells of chicken (Krek and Nigg, 1991a; 1991b) and mouse (Norbury et al., 1991). Mutation of both Thr14 and Tyr15 to nonphosphorylatable residues induced premature mitotic events in chicken. However, mutation of Thr14 by itself could not produce this phenotype while mutation of Tyr15 alone did partially induce premature mitosis, indicating that dephosphorylation of both sites was required for full activation of the vertebrate $p34^{cdc2}$ kinase. It has been suggested that since Thr14 and Tyr15 are both located in the putative ATP-binding site of $p34^{cdc2}$ protein kinase therefore phosphorylation of the two residues might reduce the binding of ATP or the efficiency of transfer of phosphate from ATP to substrate proteins (Gould and Nurse 1989; Krek and Nigg, 1991b). Another putative phosphorylation site identified in $p34^{cdc2}$ is a threonine residue, Thr167 in fission yeast, Thr161 in vertebrates (Ducommun et al., 1991; Gould et al., 1991; Krek and Nigg, 1991a; 1991b; Lorca et al., 1992). Unlike Thr14 and Tyr15, phosphorylation of this residue is required for $p34^{cdc2}$ kinase activity, and this phosphorylation can be detected during mitosis. It is

also reported that G2-phase phosphorylation of Thr161/167 is essential for MPF kinase activity and appears to be dependent upon prior association of $p34^{cdc2}$ with a cyclin B (Solomon et al., 1990; 1992). The only residue that might be phosphorylated in G1 phase is Ser277 of chicken $p34^{cdc2}$. Phosphorylation of Ser277 increases during G1 and declines on entry into S phase and may be involved in the G1/S transition, although it has not been detected in mammalian cells.

Each of the four phosphorylation sites described above have been identified in vertebrate $p34^{cdc2}$. Since Thr14 has not been identified in fission yeast (Gould et al., 1991) and Ser277 has not been recognised in other systems (Draetta and Beach, 1988, Norbury and Nurse, 1992), Tyr15 and Thr161/167 are considered to be the two most widely employed regulation sites on $p34^{cdc2}$ (Gould et al., 1991) with the interesting reservation that budding yeast does not need Tyr at 15, and therefore does not need the potential for phosphorylation at this point, to be able to hold $p34^{CDC28}$ inactive if DNA replication is incomplete.

1.5. $p13^{suc1}$, a binding protein of $p34^{cdc2}$

To investigate how the $p34^{cdc2}$ protein kinase activity is regulated at G1/S and G2/M transitions, it is necessary to identify gene products which interact with the $p34^{cdc2}$ protein kinase (Maller et al., 1989).

One protein that interacts with $p34^{cdc2}$ kinase is $p13^{suc1}$, a 13 kDa peptide of 113 amino acids encoded by the *suc1* gene of *S. pombe*. The *suc1* gene was initially identified by study of suppressors of cdc2 mutants (Hayles et al., 1986; Hindley et al., 1987). Deletion of this gene is lethal, due to a block at the anaphase-telophase transition that leaves cells with condensed chromosomes, elongated spindles and persisting activity of $p34^{cdc2}$ kinase (Hayles et al., 1986). This result indicates that the *suc1* gene product is required at a late stage in mitosis and plays a role in the inactivation of the $p34^{cdc2}$ protein kinase (Hayles et al., 1986; Moreno et al., 1989a). $p13^{suc1}$ can bind *in vitro* to $p34^{cdc2}$ from *S. pombe*, HeLa, *Xenopus* and plants (Brizuela et al., 1987; Labbe et al., 1989; John et al., 1991). Interestingly, high concentrations of $p13^{suc1}$ can, *in vitro*,

stabilise the activity of certain mutant forms of $p34^{cdc2}$ that could otherwise be thermally inactivated. This complementation *in vitro* presumably mimics the *in vivo* complementation of *cdc2*, which first revealed the *suc1* gene. Homologue(s) of the $p13^{suc1}$ protein detected in human cells were found to complex with the $p34^{cdc2}$ protein (Draetta and Beach, 1989). Cloning analysis revealed that the human proteins are smaller, of 9 kDa, and that there are two encoded by *CKS Hs1* and *CKS Hs2* (Richardson et al., 1990). Also, a homologue of the *suc1* gene, designated *CKS1* has been identified in *S. cerevisiae*, again by its capacity to suppress some *CDC28* mutations. *CKS1* encodes an 18 kDa protein peptide that shares 67% amino acid sequence identity with $p13^{suc1}$ and is physically associated with the CDC28 protein kinase. Disruption of the *CKS1* gene is lethal (Hadwiger et al., 1989; Reed et al., 1989). In *Xenopus* extracts, abundance of yeast $p13^{suc1}$ affects tyrosine phosphorylation and $p34^{cdc2}$ kinase activity of MPF (Dunphy and Newport, 1989). These findings taken together suggest that $p13^{suc1}$ functions as a regulatory binding protein in physical association with $p34^{cdc2}$ kinase and may facilitate its interaction with other proteins, especially at mitotic anaphase (Hayles et al., 1986; Booher et al., 1989).

1.6. Cyclins associate with $p34^{cdc2}$ as regulatory proteins

Cyclins are another type of regulatory protein that bind with $p34^{cdc2}$ protein and do so more tightly than does $p13^{suc1}$. Cyclins were originally discovered in the oocytes and embryos of marine invertebrates, where they undergo dramatic fluctuation in level through the cell cycle (Evans et al., 1983; Rosenthal et al., 1980). Early biochemical investigations of the cell cycle in sea urchin eggs with synchronously dividing nuclei demonstrated that most proteins were synthesised continuously but one prominent protein appeared at prophase and disappeared at each metaphase-anaphase transition, and was therefore designated cyclin. The unusual behaviour and abundance of the cyclin protein(s) in fertilised eggs allowed cloning of cyclin cDNA which was then used to make cyclin mRNA *in vitro*. When microinjected into immature *Xenopus* oocytes, the cyclin mRNA (cyclin A mRNA) was able to induce the oocytes to go through meiosis.

Meanwhile, study of MPF from frog oocytes confirmed that the cdc2 gene product $p34^{cdc2}$ is the active component that could cause maturation in unfertilised eggs (Masui and Markert, 1971; Smith and Ecker, 1971). In purified *Xenopus* MPF, $p34^{cdc2}$ was found in complex with a 45 kDa protein (Gautier et al., 1988; Lohka et al., 1988) and starfish egg MPF has been purified as a complex of a 62 kDa protein with $p34^{cdc2}$ (Labbe et al., 1988). The 45 kDa protein of *Xenopus* MPF and the 62 kDa component of starfish MPF have been identified as B-type cyclins that are required for the mitotic activity of $p34^{cdc2}$ (Minshul et al., 1989; Gautier et al., 1990).

Mutational investigation of cell cycle regulation in *S. pombe* identified a *cdc13* gene function which is involved in regulation of $p34^{cdc2}$ protein kinase. Over-expression of $p34^{cdc2}$ allowed a *cdc13* mutant to grow at its restrictive temperature, which suggested interaction of the two proteins (Booher and Beach, 1987) and *cdc13-177* mutant cells were found to be arrested with terminal phenotype characteristics of both G2 and mitosis, with condensed chromosomes but an interphase cytoplasmic array of microtubules typical of G2 (Hagan et al., 1988). Sequencing of the *S. pombe cdc13* gene has identified that it encodes a 56 kDa protein and has significant homology to cyclin B (Booher and Beach, 1988). Physical association of the $p56^{cdc13}$ and $p34^{cdc2}$ is required for both activation of $p34^{cdc2}$ kinase at mitosis and inactivation of $p34^{cdc2}$ kinase on exiting from mitosis (Moreno et al., 1989b).

In budding yeast, investigation of the *CDC28* function has revealed a role at both START and the G2/M transition. B-type cyclins (CLB1, CLB2, CLB3 and CLB4) have been isolated and considered to be involved in activation of CDC28 at mitosis. The activation of CDC28 at START requires the presence of at least one of a set of three G1 cyclins encoded by the *CLN1*, *CLN2* and *CLN3* genes (Reed et al., 1989; Lew et al., 1992) that have structures distinct from those of cyclin A and B. All cyclins have a region of about 100 amino acids, termed the cyclin box, within which they have some similarity although elsewhere they may be highly divergent. Two related genes *CLN1* and *CLN2* were detected by their ability to complement the START function of some *CDC28* mutants when present at higher concentrations, as resulted when a cDNA library was screened. The sequences of the *CLN1* and *CLN2* genes have been isolated from a

genomic DNA library and encode proteins that are highly homologous at the level of primary structure with 57% identity over the entire sequence and 72% identity over their amino terminal 50% (Reed et al., 1989). The abundance of *CLN1* and *CLN2* gene products is cell cycle regulated, reaching maximal level in late G1 (Wittenberg et al., 1990). Gene disruption of either *CLN1* or *CLN2* singly confers no obvious phenotype, which suggests that *CLN1* and *CLN2* products are functionally identical. However, double mutant cells lacking *CLN1* and *CLN2* are abnormally large and spherical as is typical of G1 cell cycle defects (Reed et al., 1989). The G1 cyclins were first discovered because of the effect of a dominant mutation WHI1 (Sudbery et al., 1980) of CLN3 which removed the proteolysis initiation (PEST) region and lead to accumulation in early G1 of levels normally attained only in later G1 with resulting advance of START and therefore smaller cell size, while inactivation of CLN3, which also differs in being continuously expressed through the cell cycle (Wittenberg et al., 1990), results in late START and large cell size (Nash et al., 1988; Reed et al., 1989; Cross, 1988). Because of the multiple G1 cyclins in *S. cerevisiae*, cells containing inactivated alleles or an extra copy of any one or two *CLN* genes are still able to traverse through START, although at altered cell size (Richardson et al., 1989). However, cells with all three *CLNs* mutated are unable to go through START and die in G1 (Watson et al., 1992). It appears that mitotic cyclins (B-type cyclins) regulate the G2-M kinase activity of cdc2/CDC28 protein, while the *CLN1*, *CLN2* and *CLN3* gene products are associated with its G1 activity. The timing of this G1 activity, if it is indeed the trigger for START, is important for maintaining the normal relationship between cell growth and division by initiating division events at relatively constant size over a range of growth rates. Lew et al. (1992) recently claimed that the three G1 cyclins in *S. cerevisiae* might function differently at the START event of mother cells (previously divided) or daughter cells (newly formed from bud), with the *CLN3* gene product being used in mother cells and the *CLN1* and *CLN2* gene product being used in daughter cells. However Linskens et al (1993) have recently shown that Cln3 functions in both mother and daughter cells of *S. cerevisiae*. Tyers et al (1992) have also suggested that Cln3 may be an upstream activator of the Clns1 and 2 that may more directly catalyse START. It is hypothesised

that active CDC28 may enhance transcription of G1 cyclins, thus a positive feedback occurs when the CDC28 protein begins to be activated at START. In addition, a possible G1 cyclin puc1 has been identified in the fission yeast (Forsburg and Nurse, 1991); however it has not been demonstrated that the execution point for puc1 is in G1 phase, and therefore it may be a different class of cyclin.

In higher eukaryotes, putative G1 cyclin genes have been identified as unlike B cyclins because they lack a destruction box close to the amino terminal and as resembling G1 cyclins because they alternatively do have PEST destruction sequences at their carboxyterminal end (Matsushime et al., 1991). Similarly, at least two sorts of G1 cyclin including cyclin C, D, and E have also been isolated in human cells (Xiong and Beach, 1991; Xiong et al., 1991; Dulic et al., 1992). The specific functions of each G1 cyclin have yet to be identified.

1.7. A regulatory network determining the timing of mitosis initiation

Both $p13^{suc1}$ and $p56^{cdc13}$ can associate with the cdc2 gene product $p34^{cdc2}$ as a physical complex, of which $p34^{cdc2}$ protein kinase is the central catalytic subunit. $p13^{suc1}$ may have roles in promoting the $p34^{cdc2}$ kinase inactivation (Nurse, 1990), although it is neither an inhibitor nor a substrate of the $p34^{cdc2}$ protein kinase. $p56^{cdc13}$ is a B-type cyclin which is required both before and during mitosis and can be phosphorylated by the $p34^{cdc2}$ protein kinase (Lehner and O'Farrell, 1989; O'Farrell et al., 1989). However, $p56^{cdc13}$ amount does not determine the timing of mitosis as indicated by the lack of effect of additional copys of the cdc13 gene (Nurse, 1990). This correlates with the activation of $p34^{cdc2}$ being determined by a change in phosphorylation as discussed earlier (Booher et al., 1989).

At least five further genes have been identified as acting together in a network that regulates the phosphorylation and therefore the activation of $p34^{cdc2}$ kinase in fission yeast. The first such gene isolated was *wee1*, which was identified by studying a class of "wee mutants" that entered mitosis prematurely. Deletion of the *wee1* gene

causes advance of cells into mitosis (Nurse, 1975; Nurse and Thuriaux, 1980), whilst over-expression of *wee1* results in cell cycle arrest at the G2/M boundary (Russell and Nurse, 1987a). This indicates that the *wee1* gene normally exerts a negative regulation of the *cdc2* gene and is dose-dependent (Maller et al., 1989; Lundgren et al., 1991). Sequence analysis of the *wee1* gene has demonstrated that it encodes a protein of 112 kDa (Russell and Nurse, 1987a). Since its apparent molecular weight on electrophoresis is 107 kDa, it is therefore often referred to as $p107^{wee1}$ (Featherstone and Russell, 1991). $p107^{wee1}$ contains the consensus sequence for a serine/threonine protein kinase, which suggests the potential for both stimulatory and inhibitory phosphorylation of $p34^{cdc2}$ protein kinase (Maller et al., 1989). The predicted biochemical role of *wee1* has been confirmed by enzymatic analysis of the over-expressed product (Featherstone and Russell, 1991). $p107^{wee1}$ is a protein kinase that *in vitro* can phosphorylate the cdc2 protein on tyrosine residue 15 (Lundgren et al., 1991). A second gene with this function named *mik1* (mitosis inhibitory kinase) has also been isolated in *S. pombe*. It encodes a 66 kDa kinase and can rescue an *S. pombe cdc2-3w wee1-50* double mutant that otherwise has minimal $p34^{cdc2}$ phosphorylation, or can complement mutations in *wee1*. The mik1 protein shares 48% amino acid identity with the wee1 protein over a 214 amino acid protein kinase catalytic domain (Lundgren et al., 1991). Deletion of *mik1* does not confer a *wee* phenotype nor rescue a *cdc25*$^-$ mutant. However, the double mutant of *wee1*$^-$ and *mik1*$^-$ does display a wee phenotype at the normally permissive temperature for *wee1-50* and is lethal at the restrictive temperature. It has been shown that wee1 protein is capable of tyrosine autophosphorylation *in vitro* (Featherstone and Russell, 1991), even though both wee1 and mik1 proteins are more closely related in structure to the family of serine/threinine kinase than those generally associated with tyrosine specificity (Hanks et al., 1988), suggesting that wee1 is a new class of cell cycle specific protein kinase. Combined genetic and biochemical experiments indicate that in the absence of wee1 and mik1 activty, $p34^{cdc2}$ rapidly loses the phosphate groups on tyrosine residues, both in yeast strains undergoing mitotic lethality and in those that are viable owing to a compensating mutation within cdc2. It has therefore been suggested

that wee1 and mik1 act redundantly and cooperatively on cdc2 (Lundgren et al., 1991; Featherstone and Russell, 1991).

A key positive regulatory element in the network is the *cdc25* gene product, which is an 80 kDa protein required for activation of $p34^{cdc2}$ protein kinase and entry into mitosis. Deletion of the *cdc25* gene is lethal in cells that are otherwise genetically normal. Temperature sensitive *cdc25* mutant cells of *S. pombe* can go through a cell cycle normally at 25°C, but arrest uniformly at the G2/M boundary at restrictive temperature (Fantes, 1979; King and Hyams, 1982; Alfa et al., 1989). The $p34^{cdc2}$ protein kinase in *cdc25*$^-$ arrested cells is phosphorylated and its kinase activity remains low, as in interphase (Nurse, 1990). Soon after shifting to the permissive temperature, the $p34^{cdc2}$ kinase becomes dephosphorylated and a consequent peak of catalytic activity results in cells entering mitosis (Moreno et al., 1989b). Over-expression of *cdc25*, however, causes a wee phenotype with premature entry into mitosis (Maller, 1991). This indicates that the function of the *cdc25* gene product is dose-dependent and positively involved in regulating the onset of the mitosis by triggering dephosphorylation, and therefore activation, of the cdc2 protein kinase.

It has been found that *cdc25*-like genes have been widely conserved in eukaryotes. Functional homologues of *cdc25* have since been isolated from diverse organisms; such as the *MIH1* gene from *S. cerevisiae* (Russell et al., 1989), *string* from Drosophila (Edgar and O'Farrell, 1989; Alphey et al., 1992), cdc25 from *Xenopus* (Kumagai and Dunphy, 1992), and *Hscdc25* from human cells (Sadhu et al., 1990). These genes have been found to be able to rescue the *S. pombe cdc25-22* mutant.

The fission yeast *cdc25* gene encodes a 80 kDa protein bearing no extensive similarity to most of the well-known phosphatases, but containing a cysteine residue that is essential for catalysis and is conserved in most of the known tyrosine phosphatases (Dunphy and Kumagai, 1991; Gautier et al., 1991). The level of $p80^{cdc25}$ protein increases in *S. pombe* cells proceeding through G2 and it peaks just before the onset of mitosis (Ducommun et al., 1990). Recent investigations have established that $p80^{cdc25}$ protein is essentially a specific protein phosphotyrosine phosphatase (PTPase). This finding has been supported by observations that inactivation of cdc25 results in a late G2

cell cycle arrest, in which $p34^{cdc2}$ becomes maximally phosphorylated on Tyr-15 (Gould and Nurse, 1989) and that there is no requirement for cdc25 gene function in a cdc2 mutant that has nonphosphorylatable phenylalanine at position 15 (Gould et al., 1990; Millar and Russell, 1992). Studies in *Xenopus* show that $p80^{cdc25}$ protein made in *E. coli* displays specific tyrosine phosphatase activity toward Tyr15 on $p34^{cdc2}$ in the absence of any other eukaryotic cell components (Smythe and Newport, 1992). All these findings suggest that cdc25 has a key role in determining the timing of $p34^{cdc2}$ kinase activation and thus of mitotic onset (Nurse, 1990).

Another gene that has been identified as a member of the cdc2 control network in *S. pombe* is *nim1* (also known in its complete form as *cdr1* (Russell and Nurse, 1987b Feilotter et al., 1991)) which can also generate a wee phenotype if it is present in several copies per cell (Russell and Nurse, 1986, 1987b). The *nim1* gene product is a 50 kDa protein that closely resembles known protein kinases and induces mitosis by acting as a negative regulator of the *wee1* gene product in a dose-dependent manner (Russell and Nurse, 1987b). Recent investigations by Coleman et al. (1993) have provided direct biochemical evidence for the down-regulation of wee1 activity by nim1. They have demonstrated that the nim1 protein kinase inhibits the wee1 kinase by directly phosphorylating both tyrosine and serine residues at its C-terminal region and leads to a substantial decline in wee1 activity toward the $p34^{cdc2}$ protein.

Studies using combinations of mutants of *cdc2*, *wee1*, *mik1*, *nim1* and *cdc25* genes in yeasts have led to the proposal (Russell and Nurse, 1987b; Nurse, 1990) that these five gene functions are organised in a regulatory network in which activation of the key $p34^{cdc2}$ kinase and therefore the timing of mitosis is regulated by two pathways. *wee1/mik1* and *nim1* contribute to a pathway in which *wee1/mik1* acts negatively to inhibit activation of $p34^{cdc2}$ by promoting inactivation of the kinase by phosphorylation of its tyrosine residues and in mammalian cells also its Thr14 residue. The cdc25 pathway then positively regulates the activation of $p34^{cdc2}$ protein kinase by directly dephosphorylating Tyr15 on the kinase which then leads to initiation of mitosis. In mammalian cells Thr14 phosphate is also present and the set of mammalian cdc25 enzymes removes it as well. The *nim1* gene product, however, inhibits the *wee1/mik1*

function, which therefore has the effect of a positive regulator of $p34^{cdc2}$ protein kinase. All these genes interact through the two pathways or cascades of the regulatory network to modulate the $p34^{cdc2}$ kinase activity and adjust the timing of mitosis under the influence of cell size and growth rate. The molecular mechanisms by which the latter two parameters are perceived and influence the cascades is still unknown.

1.8. Regulation of the G1/S transition

Regulation of the G1/S transition is currently even less well understood than that of the G2/M transition, although the $p34^{cdc2/CDC28}$ protein functions at both control points. Jacobs has suggested the possibility that a universal S phase promoting factor (SPF), that is as phylogentially conserved as the "maturation or mitosis promoting factor (MPF)", could drive yeast, vertebrate and even higher plant cells from G1 phase into S phase (Jacobs, 1992). However, the yeasts have single $p34^{cdc2/CDC28}$ proteins that must perform both functions whereas higher eukaryotes have a cdk2 variant that acts exclusively at G1/S and in complex with cyclin A (Pines and Hunter, 1990).

Components that might constitute an SPF complex have been identified in different organisms. The functional differences of *cdc2/CDC28* products at START and at the G2/M transition in yeasts might depend on the type of cyclins that they associate with. While mitotically active MPF contains B-type or mitotic cyclins, the START function in budding yeast is mediated by a G1 cyclin-$p34^{CDC28}$ complex. The three known G1 cyclins (CLN1, CLN2, and CLN3) identified in budding yeast bear biochemical similarities to mitotic cyclins (Tyers et al., 1992) and associate with $p34^{CDC28}$ at START. This is supported by the evidence that over-expression of the *CLN* genes advances cells prematurely into S phase and that the CLN-$p34^{CDC28}$ complex has protein kinase activity peaking in late G1 phase (Diri and Nasmyth, 1991; Wittenberg et al., 1990; Tyers et al., 1993). Recent studies have suggested that the level of G1 cyclins is transcriptionally regulated in budding yeast. The CLN3-$p34^{CDC28}$ activity acts through the phosphoprotein transcription factors *SWI4* and *SWI6* which, when phosphorylated by the kinase, promote the transcription of *CLN1* and *CLN2*, which

in turn leads by positive feedback to an increase in CLN-p34^{CDC28} activity, therefore more SWI4 and SWI6 are phosphorylated and activated (Nasmyth and Dirick, 1991; Dirick et al., 1992; Tyers et al., 1993). CLN3 may behave differently since its level does not fluctuate through the cycle and the activity of CLN3-p34^{CDC28} does not oscillate. However CLN3 can activate transcription of at least five other cyclins and may therefore be considered as a cyclin that indirectly acts at START by activating other downstream G1 cyclins that are directly involved at late G1/S phase (Tyers et al., 1993).

In higher eukaryotes, DNA replication and mitosis seem to be controlled by different cdc2-like proteins that bind different cyclins (Pines and Hunter, 1990; Elledge and Spottswood, 1991; Paris et al., 1991; Tsai et al., 1991; Meyerson et al., 1992; Fang and Newport, 1991; Devoto et al., 1992). In both *Xenopus* and *Drosophila* cells, for example, a cdc2 and a close variant cdk2 protein have been identified (Dunphy et al., 1988; Dunphy and Newport, 1989; Lehner and O'Farrell, 1990; Paris et al., 1991). Similarly more than one cdc2 related protein has been characterised in human cells (Pines and Hunter, 1990; Hirayama et al., 1991). The two cdc2 related proteins, of 32 kDa and 34 kDa, in *Xenopus* eggs both have cyclin-dependent kinase activity and share 66% homology of amino acid sequence with each other, but they appear to act differently during the cell cycle. The 32 kDa cdc2 variant is named cdk2 kinase because it is cyclin dependent and is closely related to the *cdc2* gene product p34^{cdc2} (Blow and Nurse, 1990; Norbury and Nurse, 1992). The cdk2 kinase is physically associated with cyclin A, rather than cyclin B, and with a novel doublet of 45 kDa proteins that may regulate its activity, which is higher during S phase than at mitosis. In contrast, the cyclin B-cdc2 protein kinase activity does decrease dramatically in early mitosis. Depletion of the cdk2 kinase from a *Xenopus* extract blocks DNA replication, but does not inhibit entry into mitosis (Fang and Newport, 1991). Whereas, depletion of the 34 kDa cdc2 homologue, or the absence of mitotic cyclins from an extract, does not inhibit DNA replication, but does block the onset of mitosis (Fang and Newport, 1991, Norbury and Nurse, 1992). These findings suggest that in *Xenopus* eggs, the cyclin A-cdk2 kinase complex regulates the G1/S transition and/or is required during S phase, while a cyclin B-p34^{cdc2}-like kinase complex controls the initiation of mitosis.

In mammalian cells more than one complex with cdc2-like proteins probably exists in late G1 phase. A key component of one late G1 complex is a nuclear phosphoprotein retinoblastoma protein (RB), which can inhibit progression of cells into S phase (Goodrich et al., 1991; Bandara et al., 1991). The human RB is synthesised throughout the cell cycle, but becomes highly phosphorylated at the G1/S transition when transcription of enzymes for DNA synthesis increases. In G1 phase the hypophosphorylated form of RB is associated with a transcription factor E2F, which has the potential for promoting expression of genes encoding enzymes required for DNA synthesis (Chellappan et al., 1991). When RB becomes phosphorylated at the G1/S transition, it is disassociated from the RB-E2F complex (Chellappan et al., 1991; Jacobs, 1992) and the released E2F is than able to associate in a complex of E2F-cyclinA-p107-cdk2. The complex accumulates during S phase and possesses H1 kinase activity, due to cdk2, and sequence-specific DNA binding activity, due to E2F (Devoto et al., 1992). Therefore, the cyclin A-cdk2 protein kinase complex may play an important role in regulating the G1/S transition in human cells. It may be preceded by the formation of complexes containing cyclin D and/or E, which accumulate earlier than cyclin A. These earlier complexes could therefore function, like the Cln3 complex of *S. cerevisiae* at the late G1 control point.

1.9. Regulation of exit from mitosis

Exit from mitosis has been less extensively investigated than entry into mitosis. According to the model that has now become widely accepted, high $p34^{cdc2}$ kinase activity maintains the cell in mitosis by inhibiting anaphase, and exit from mitosis requires kinase inactivation and the operation of phosphoprotein phosphatases that may remove phosphates attached during the earlier kinase activity (Nurse, 1990). Eggs of *Xenopus* that are in late prophase or metaphase of meiosis or mitosis contain an extractable cytoplasmic activity called maturation-promoting factor (MPF) that has been mentioned earlier in connection with its capacity to induce immature oocytes to progress from the early prophase in which they are normally arrested until stimulated by progesterone

(Masui and Markert, 1971). MPF from eggs that are arrested in metaphase, such as mature eggs awaiting fertilisation, when microinjected into oocytes stimulates rapid passage through the meiosis I and arrest at the second meiotic metaphase (Lohka and Masui, 1984; Gerhart et al., 1984). The peaks of $p34^{cdc2}$ kinase activity in oocytes are coincident with the peaks in level of the mitotic cyclin B which, together with $p34^{cdc2}$, comprises the functional component of the MPF extract. Cyclin B cyclically accumulates during each interphase until metaphase, then disappears when $p34^{cdc2}$ protein kinase becomes inactivated. This coupling of cyclin level with $p34^{cdc2}$ activity suggests that inactivation of $p34^{cdc2}$ kinase (MPF) depends on the disappearance of the cyclin. Experiments have shown that fluctuation of cyclin concentration is due to variation in its half-life rather than its rate of synthesis; the half-life is long in interphase and short in mitosis (Murray and Kirschner, 1989). Evans et al. (1983) and Murray (1987) found that cyclin was stable in cells that arrested in meiosis and mitosis, implying that cyclin degradation was initiated as an integral part of exit from mitosis and meiosis. Degradation of cyclin is thought to occur by attachment of ubiquitin to the "destruction box" region within the N-terminal part of the protein (Glotzer et al., 1991). A mutant cyclin lacking the "destruction box" was fully capable of activating MPF and driving entry into mitosis, but its failure to be degraded resulted in arrest at metaphase. Similarly, injection of mRNA for the mutated version of cyclin into cleaving eggs also caused metaphase arrest (Murray, 1987; Murray and Kirschner, 1989). All of these findings lead to the notion that cyclin degradation is required for inactivation of the $p34^{cdc2}$ kinase (MPF) at metaphase and triggers the metaphase-anaphase transition that leads to exit from mitosis.

Exit from mitosis also requires the presence of $p13^{suc1}$. Deletion of the *suc1* gene from *S. pombe* results in arrest in anaphase and persisting $p34^{cdc2}$ kinase activity, which reveals that an essential function of $p13^{suc1}$ is participation in $p34^{cdc2}$ inactivation that is necessary for exit from mitosis and re-entry into interphase.

Recent evidence suggests that progress through anaphase may not be universally determined simply by cyclin B as once seemed likely. Firstly the block point from failure of cyclin B breakdown may be a little later than metaphase at least in the budding yeast,

where anaphase/telophase is the time at which inactivation of $p34^{cdc2}$ kinase is necessary for normal progress (Surana et al., 1993) since the *cdc15* mutant can go through anaphase but not complete telophase in the presence of persisting high levels of CLB2/CDC28 kinase activity, becoming arrested with partially separated chromosomes and elongated mitotic spindle. Additional evidence in budding yeast has shown that over-expression of either a mutated *CLB2* lacking a "destruction box" or multiple copies of wild type *CLB2* caused wild type cells to arrest at telophase, also consistent with a block point later than metaphase. Secondly it may not be proteolysis of cyclin B that is critical in all cell types since there is some evidence that in frog eggs it is not degradation of specifically cyclin B that must occur for progress beyond metaphase. In frog egg extracts non-degradable cyclin B did not block progress to anaphase in the presence of added calcium. However, addition of a peptide containing the cyclin degradation box sequence, which competed for degradation with all cyclins containing this sequence and presumably resulted in elevation of the level of more than just cyclin B, did block chromosome separation (Holloway et al., 1993). Therefore proteolysis of some cyclin, if not cyclin B, is implicated at the metaphase/anaphase transition. Although cyclin proteolysis is necessary for completion of mitosis, the completion of mitosis does not induce cyclin proteolysis since experiments in an *esp1* (extra spindle pole bodies) mutant of budding yeast proved that destruction of CLB2/CDC28 kinase could occur in the absence of anaphase completion (Surana et al., 1993). In budding yeast, therefore, the balance of evidence suggests that CLB2/CDC28 kinase destruction is not dependent upon the completion of anaphase, but that its inactivation is required for completion of telophase and final exit from mitosis. A similar essential contribution of cyclin degradation to completion of nuclear division is probably universal.

1.10. The cell division cycle in higher plants

Cell division activity in higher plants is more localised to special tissues than in vertebrates since it is largely confined to specific areas called meristems, from which new organs can be formed by cell proliferation throughout the whole life of the organism.

Meristems in root, shoot and leaf each behave slightly differently in cell division response during differentiation and in response to various plant hormones. However, there are reasons to think that the fundamental control of the cell cycle in higher plants may be similar to that in the model yeast systems.

Investigation of cell cycle control in higher plants was pioneered by Van't Hof (1973, 1985) who first established that control points were located somewhere in G1 and G2 phase rather than in S and M phases of the plant cell cycle. By studying the effects of sucrose starvation, gamma irradiation and inhibitors of DNA synthesis on cultured root meristems, he was able to arrest meristematic cells in G1 or G2 phases, which led to the supposition that plant cells could have two principal control points in the cell cycle: One was postulated to reside in late G1 phase and perhaps determine whether cells are ready to exit from G1 phase and initiate DNA replication; the other was postulated to be located in late G2 phase and to determine the onset of mitosis. The implication was that to complete a cell cycle, cells must be competent to pass through the two principle control points although there was no evidence for a single control point in each phase nor any indication where they might be located (Van't Hof and Kovacs, 1970; Van't Hof, 1973). We now have more direct data in support of the hypothesis of late G1 and G2 phase control points in higher plants, and also indications that these are functionally equivalent to the two major transitions in yeasts, the START control in late G1 and the G2/M transition in late G2. Biochemical and genetic evidence for plant cell division regulating proteins, which are equivalent to those that function at both control points in yeast, did not emerge until the 1980's. Stratton and Trewavas (1981) first detected the presence of a G2/M-associated histone H1 kinase activity that is cAMP and Ca^{+2}-independent in plants. However it is difficult to assess whether any part of this was $p34^{cdc2}$ since John, Sek and Hayles (1991) showed that plant meristem cells contain several such enzymes, and H1 histone is a suitable substrate for many kinases. Plant histone H1 kinase activity was later linked to the widely conserved key cell division regulating protein $p34^{cdc2}$ by work carried out in a couple of laboratories. The first link was made by John, Sek and Lee (1989). Using an antibody against the PSTAIR peptide sequence, which is specific for $p34^{cdc2}$-like proteins, they identified a 34 kDa protein in unicellular algae as well as dicotyledonous and

monocotyledonous plants. Affinity purified $p34^{cdc2}$ enzyme containing this sequence was shown to have cAMP and Ca^{+2}-independent histone H1 kinase activity and specificity for the peptide ADAQHATPPKKKRKVEDPKDF that is characteristic of $p34^{cdc2}$ (John et al., 1991; 1992). Protein extracts from pea have also been found to possess a 34 kDa component which reacts with a monoclonal antibody directed against an unknown region of the fission yeast $p34^{cdc2}$ (Feiler and Jacobs, 1990). More recently similar $p34^{cdc2}$ activities have been found in maize (Colasanti, 1991) and alfalfa (Magyar et al., 1993).

The presence of $p34^{cdc2}$ homologues in plant cells is further supported by molecular and genetic evidence that higher plant cells contain homologues of the fission yeast cdc2 gene that have retained its essential functions since they can complement yeast cells with mutant cdc2 or CDC28. It is therefore highly plausible that the activities performed in yeast cells are among the natural functions of the plant cdc2 homologue proteins. Homologues of *cdc2/CDC28* have now been cloned by polymerase chain reaction (PCR) and degenerate oligonucleotide probing from pea, *Arabidopsis*, alfalfa, maize and soybean (Colasanti et al., 1991; Feiler and Jacobs, 1991; Ferreira et al., 1991; Hirayama et al., 1991; Hirt et al., 1991; Miao et al., 1993). Of the cloned plant cdc2 genes, the maize and alfalfa *cdc2* homologues have been found to be able to rescue *S. cerevisiae cdc28-1N* and *S. pombe cdc2-33* mutants respectively. Cloning of cdc2 homologues has established that both the *Arabidopsis* and soybean genomes contain at least two *cdc2*-like genes. The two cloned soybean *cdc2*-like genes share 90% sequence homology in the coding region and both can rescue a *cdc28* mutation in *S. cerevisiae*, whereas the *Arabidopsis cdc2* homologues can only partially complement the *S. pombe cdc2* mutant (Hirayama et al., 1991; Miao et al., 1993). Genomic Southern-blot analysis with the two *Arabidopsis cdc2* homologue cDNA probes suggests that the *Arabidopsis* genome contains several additional *cdc2*-like genes which, together with the two cloned *cdc2*-like genes, may constitute a cdc2 gene family (Hirayama et al., 1991). Multiple *cdc2* gene homologues have also been detected in maize by Southern-blot analysis (Colasanti et al., 1991).

In addition to the $p34^{cdc2}$ homologue, a homologue of the $p34^{cdc2}$ binding protein $p13^{suc1}$ has also been detected in wheat, pea and *Chlamydomonas* using a polyclonal antibody against *S. pombe* $p13^{suc1}$ (John et al., 1991). Moreover, polymerase chain reaction and degenerate oligonucleotide probing have yielded several cyclin-like genes, including one clone from carrot, two from soybean and three from pea (Hata et al., 1991; reviewed by Jacobs, 1992). However, the type of the cyclins and their relationship with $p13^{suc1}$ and possible multiple forms of $p34^{cdc2}$-like protein kinase in the higher plant cell cycle have not yet been determined. Plant cyclins do not conform very distinctly to the cyclin families ABCDE of animal cells.

The finding that more than one type of $p34^{cdc2}$-like protein kinase is present in both higher plants and vertebrate animals suggests that cell cycle mechanisms in multicellular organisms are more complex than in yeast. Some special complexity may be peculiar to the plant kingdom, in which cell division occurs in the absence of a particulate centrosome and in the presence of the pre-prophase band (PPB) of microtubules, which is not observed in either the yeasts or animal cells. The PPB appears at pre-prophase of the cell cycle and disappears at metaphase, and it is involved in determining the site of the cell plate on completion of cell division. Immunocytochemical studies have demonstrated the presence of $p34^{cdc2}$-like (PSTAIR-containing) protein in the region of the PPB (Mineyuki et al., 1991; John and Wu, 1992; Colasanti et al., 1993). In maize 10% of PPB stain positively with antibody specific for $p34^{cdc2}$ rather than for the possibly diverse group of PSTAIR containing proteins (Colasanti et al., 1993), which has led to the hypothesis of its possible involvement in the determination of cell plate formation during the plant cell division. However there is evidence that the involvement of $p34^{cdc2}$ with the PPB may be in triggering its depolymerisation at anaphase. Evidence that okadaic acid blocks events, such as spindle formation and chromosome condensation, that have been considered possible signals for PPB breakdown, while the inhibitor does not block $p34^{cdc2}$ activation, which is coincident with PPB breakdown, has lead John and Wu (1992) and John et al. (1993) to propose that activation of $p34^{cdc2}$ is the trigger for PPB breakdown.

Cell division in higher plants is restricted to meristems, where cells are generated with the specific orientations that allow subsequent tissue formation while retaining the original relative cell positions (Gunning, 1980). The level of cdc2 expression is higher in meristematic tissues. For example, mitotic activity in the seedling wheat leaf is restricted to the lower 8 mm and coincides with maximum levels of $p34^{cdc2}$-like protein detected by PSTAIR antibody (John et al., 1990) and with the occurrence of $p34^{cdc2}$-like protein kinase activity recovered by affinity purified $p13^{suc1}$ (John et al., 1993). Moreover, different plant $p34^{cdc2}$-like genes can have alternative expression levels in different tissues such as root or shoot meristems. This could indicate that plant cell division in different tissues may be controlled by different $p34^{cdc2}$-like proteins which could respond to different signals including phytohormones. This kind of situation has been detected in soybean in which two cloned cdc2-like genes were expressed at different levels in root and shoot (Miao et al., 1993). The sharp restriction of plant cdc2 gene transcription and protein synthesis to meristem cells is probably more than coincidental. For example, cells leaving the meristem can then increase beyond sizes that would allow them to divide if located within the meristem but they do not divide and reduced levels of the key division protein commit them alternatively to differentiation (John et al, 1993).

1.11. The cell cycle of *Chlamydomonas*

Chlamydomonas differs from many eukaryotes by producing 2, 4, 8 or 16 daughter cells under conditions of increasing growth rate. However, functional similarity between division control in *Chlamydomonas* and division control in yeast has been clarified by investigating the location of control points of cell division and analysis of the mechanism by which different growth rates cause different division numbers (John, 1984).

An extended G1 phase is present and its duration is stabilised against moderate changes in temperature and growth rate. The overall circadian duration of the cell cycle at moderate to fast growth rates and the capacity for temperature compensation in the range of 20°C to 30°C has led to recognition of a biological timer as the controller of G1

phase duration (John, 1984, 1987). Cells grow up to a point in late G1 at which they can become committed to divide and thereafter will carry through the processes of DNA synthesis, mitosis and cytokinesis even if net growth is completely prevented by darkness or absence of CO_2 (Donnan et al., 1985). The first commitment therefore divides the cell cycle of *Chlamydomonas* into two very different time periods. The G1 phase before first commitment is variable and growth dependent, whereas the portion of cell cycle after the first commitment, in which cells undergo duplication of DNA and nuclei then cell number, is relatively constant and little influenced by growth. This difference has led to a consideration that two types of control processes, a timer and then a size based control, are involved in the cell cycle of *Chlamydomonas* (Donnan et al., 1985; McAteer et al., 1985).

Both timer (cell age) and cell size can influence the occurrence of commitment to division, which terminates G1 phase. Cell must have passed a sufficient time, have current on-going growth and also have attained a size capable of forming at least two daughter cells before commitment to division will occur. Of the two requirements, size and age, whichever is last attained becomes the rate-limiting factor in commitment to divide (John, 1987). At very slow growth rates (mean generation time > 44 h) the cell cycle of *Chlamydomonas* is identical with that of other eukaryotes and probably higher plants, because the G1 phase is prolonged after expiry of the precommitment timer since cells are not yet large enough to divide. Therefore commitment to division at very slow growth rates occurs when the size threshold is first reached. No additional commitments are appropriate, since the cell is just large enough to form two daughters. Therefore division results in two daughter cells in each cell cycle, as is common in other eukaryotes. At higher growth rates, cells exceed the minimum size by the time they are old enough for commitment, which makes the size requirement cryptic. The pre-commitment biological timer running through G1 phase has extended its duration and resulted in more than one doubling in cell mass. This is resolved by recurrence of commitments, each leading to a single round of DNA replication followed by a single mitosis and cytokinesis to produce multiple daughter cells in the 2^n series (Fig. 1.1). Experiments based on centrifugal separation of larger and smaller cells from a single

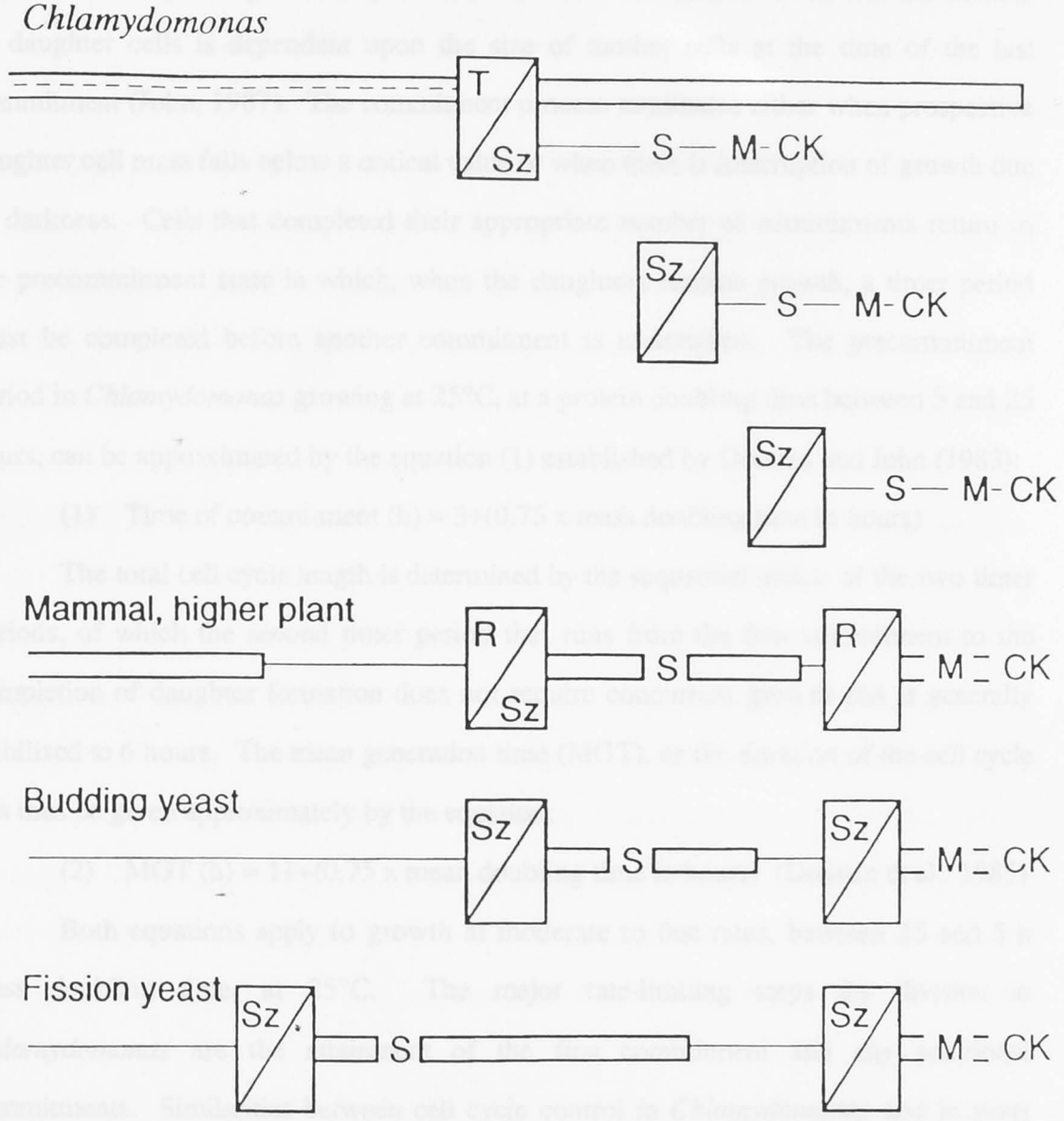


Fig. 1.1. Rate limiting control points in the cell cycles of plants and other eukaryotes. The equivalence of control points is indicated by common boxed symbols. A general requirement of the late G1/S phase transition is for a critical minimum cell size. The first such control in *Chlamydomonas* is labelled T, to signify that at higher growth rates the minimum size for entry to division is passed due to the operation of a timer ═. The longer G2 phase indicated for fisson yeast indicates the importance in that species of attaining a larger cell size for the G2/M transition. Key; T, expiry of a critical time; Sz, attainment of a critical size; R, restriction point potentially influenced by hormones; ═, timed period relatively uninfluenced by growth rate; —, period of variable duration greatly influenced by growth rate. A similarity of molecular mechanisms at these control points was first demonstrated in yeasts (Beach et al. 1982). (From John, 1984).

population and growing them in parallel at the same rate have revealed that the number of daughter cells is dependent upon the size of mother cells at the time of the last commitment (John, 1987). The commitment process terminates either when prospective daughter cell mass falls below a critical value or when there is interruption of growth due to darkness. Cells that completed their appropriate number of commitments return to the precommitment state in which, when the daughters resume growth, a timer period must be completed before another commitment is undertaken. The precommitment period in *Chlamydomonas* growing at 25°C, at a protein doubling time between 5 and 25 hours, can be approximated by the equation (1) established by Donnan and John (1983):

(1) Time of commitment (h) = 5+(0.75 x mass doubling time in hours)

The total cell cycle length is determined by the sequential action of the two timer periods, of which the second timer period that runs from the first commitment to the completion of daughter formation does not require concurrent growth and is generally stabilised to 6 hours. The mean generation time (MGT), or the duration of the cell cycle can thus be given approximately by the equation:

(2) MGT (h) = 11+(0.75 x mean doubling time in hours) (Donnan et al., 1985)

Both equations apply to growth at moderate to fast rates, between 25 and 5 h mass doubling time, at 25°C. The major rate-limiting steps for division in *Chlamydomonas* are the attainment of the first commitment and any additional commitments. Similarities between cell cycle control in *Chlamydomonas* and in yeast include (1) both commitment to division in *Chlamydomonas* and START in yeast are located in late G1 phase immediately before DNA synthesis; (2) both commitment and START require concurrent growth and a critical minimum cell size and (3) when cells have attained commitment to divide they will carry out a single doubling of DNA, mitosis and cytokinesis without further requirement for growth. Commitment to division in *Chlamydomonas* is therefore functionally equivalent to the START control point in the yeast cell cycle (John, 1984, 1987).

1.12. *Chlamydomonas* as a model system for the cell cycle control

Studies on the plant cell cycle have lagged behind those of other systems because of a lack of cell cycle mutants and the difficulty of performing experiments that seek to vary growth rates and measure cell size in higher plant tissues (Francis, 1992). The situation is now changing rapidly with the development of molecular techniques to detect the possible plant homologues of yeast cell division cycle genes, but it is still limited by the dependence of progress on prior detection of genes in yeast. This could be a severe limitation given the possibility, discussed earlier, that the regulation of some plant cell cycle events may be slightly different, or more complex than that in yeasts. Our laboratory therefore has, since the late 1980's, put effort into isolation of plant cell division cycle mutants from the unicellular plant *Chlamydomonas*, while continuing investigation into the conservation of yeast cdc gene homologues in higher plants. There are numerous advantages of using *Chlamydomonas* as a model system for the genetic analysis of the cell cycle in plants: as shown in Fig. 1.2, *Chlamydomonas* is a unicellular and haploid green plant, which can grow both on agar and in liquid media and is suitable for selection of cell division cycle mutants from large mutagenised populations. As a haploid plant, mutations in the single gene copies are immediately expressed in the phenotype and not masked by the unmodified gene product of an homologous gene, as usually occurs in diploid cells.

Mendelian analysis is also available since lack of nitrogen source in the growth medium can switch haploid vegetative cells into a sexual cycle, in which gametes with opposite mating type can be produced by mitosis (reviewed by Harris, 1989). The gametes of opposite mating type fuse to form diploid zygospores that can germinate by meiosis to yield tetrad progeny. This permits biochemical and Mendelian genetic analysis of whether the phenotypes derive from single genes.

Chlamydomonas can be grown photoautotrophically, mixotrophically or heterotrophically and their rate of growth can be manipulated by modulation of nutrition, light or CO_2 concentration. The effects on cell size and division can also be accurately

measured. In addition, *Chlamydomonas* can be readily synchronised by entrainment in alternating light and dark periods without using chemical inhibitors (Bruce, 1970) and is therefore amenable to investigation of the coupling of biochemical changes in cell cycle regulating proteins to cell cycle progress (John and Wu, 1992). Mitosis in *Chlamydomonas* involves a so called "metaphase band" of microtubules, which appears in prophase and persists during mitosis marking the future region of phycoplast formation between new daughter cells. This "metaphase band" has some structural and functional similarity to the pre-prophase band of higher plants (Doonan and Grief, 1987). Therefore study of cell cycle control using *Chlamydomonas* as a model system could provide information that is as useful as that derived from yeasts and possibly of greater relevance to higher plants.

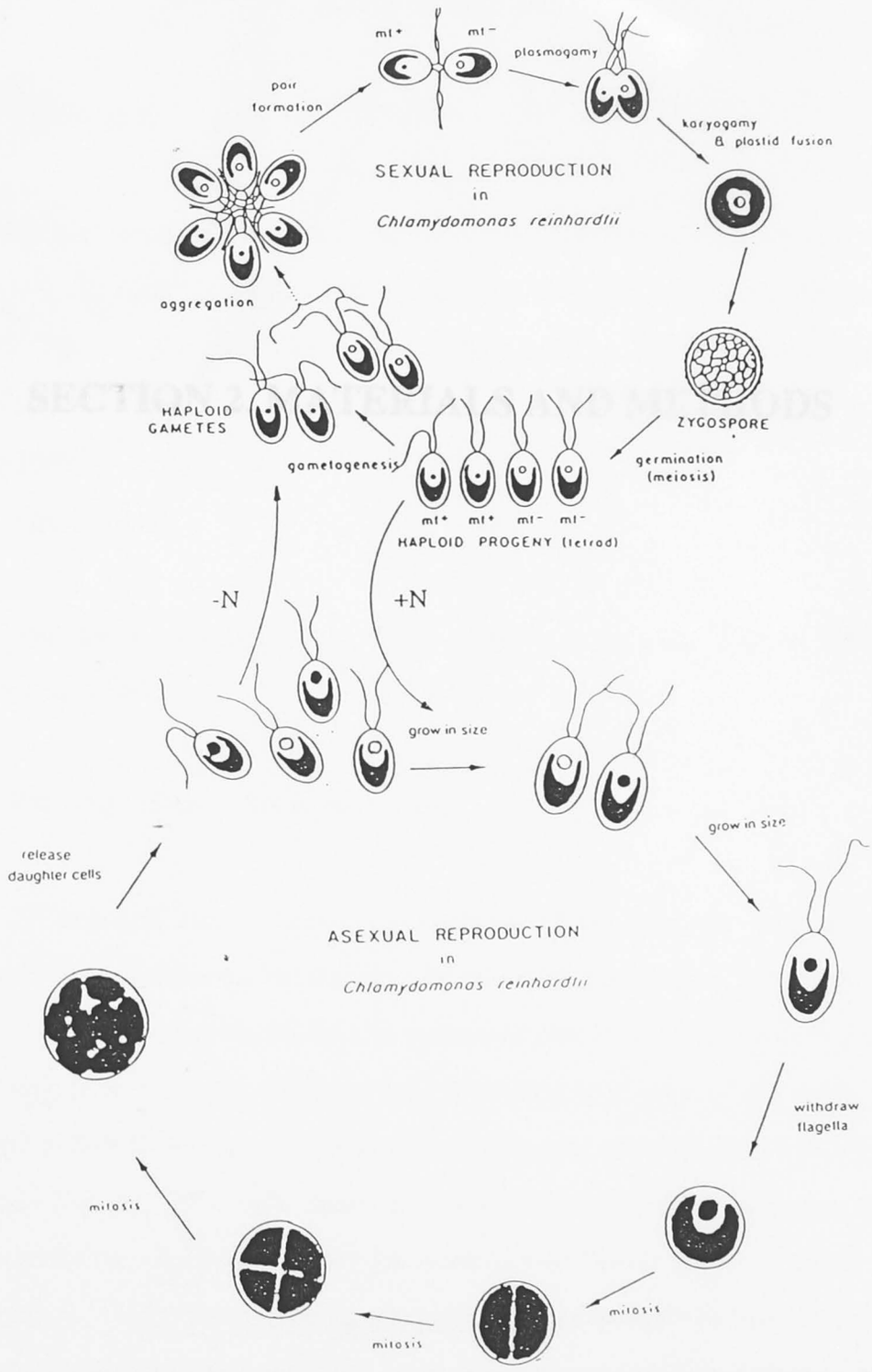


Fig. 1.2. The life cycle of *Chlamydomonas reinhardtii*. (adapted from the figure by Karen Vanwinkle-Swift in Harris, 1989).

SECTION 2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

General and analytical grade chemicals were supplied by: the Sigma Chemical Company Ltd., Ajax Chemical, Clyde Industries Ltd. Auburn Australia; Mallinckrodt Australia Pty. Ltd. Clayton, Victoria and BDH Chemicals (Australia) Pty. Ltd., Port Fairy, Victoria.

2.1.2. Equipment

A description of equipment used and its place of purchase is given where first mentioned in the text.

2.1.3. The organism, *Chlamydomonas*

The organism that was used in this analysis of the cell cycle is the unicellular green plant *Chlamydomonas reinhardtii*. In terms of classification it belongs to the genus *Chlamydomonas* in the family *Chlamydomonadaceae* (Harris, 1989). This green alga is ovoid in shape, about 10-20 μm long depending upon stage of cell cycle, with a pair of apical flagella (about 11μm long) at its anterior end nucleated from a pair of basal bodies (see Fig. 2.1). The basal bodies are connected to each other by a striated fibre which contains the calcium-modulated contractile protein centrin (Salisbury et al., 1984; Salisbury et al., 1988). The basal bodies form the focus of at least two other cytoskeletal systems composed of microtubules and actin. From the basal bodies a cruciate flagella root system extends into the cell (~~Ringo, 1967~~; Gaffal, 1988; Gaffal and el-Gammal ~~et al.~~, 1990). This root system contains acetylated tubulin and many secondary microtubules radiate from it to the cell periphery (LeDizet and Piperno, 1986; and see Fig. 2.1.2). Another cytoskeletal system is composed of actin, which forms the major microfilament

component of a fertilisation tubule in mating cells but is also present in the region of basal bodies and phycoplast (Harper et al., 1992). The single nucleus contains a haploid genome in vegetative cells and comprises 10% of the cell volume (Schötz et al., 1972) lying beneath the basal-body root complex (Ringo, 1967). A single cup shaped chloroplast surrounds the nucleus and occupies 40% of the cell volume (Schötz, 1972, see Fig. 2.1.1). The major constituents of the cell wall are hydroxyproline-rich glycoproteins, with arabinose, galactose and glucose as the predominant sugars and cellulose, a minor component, as indicated by resistance to cellulase digestion (Horne et al., 1971). In addition, *Chlamydomonas* cells contain an eyespot, Golgi bodies and mitochondria as shown in Fig. 2.1.1.

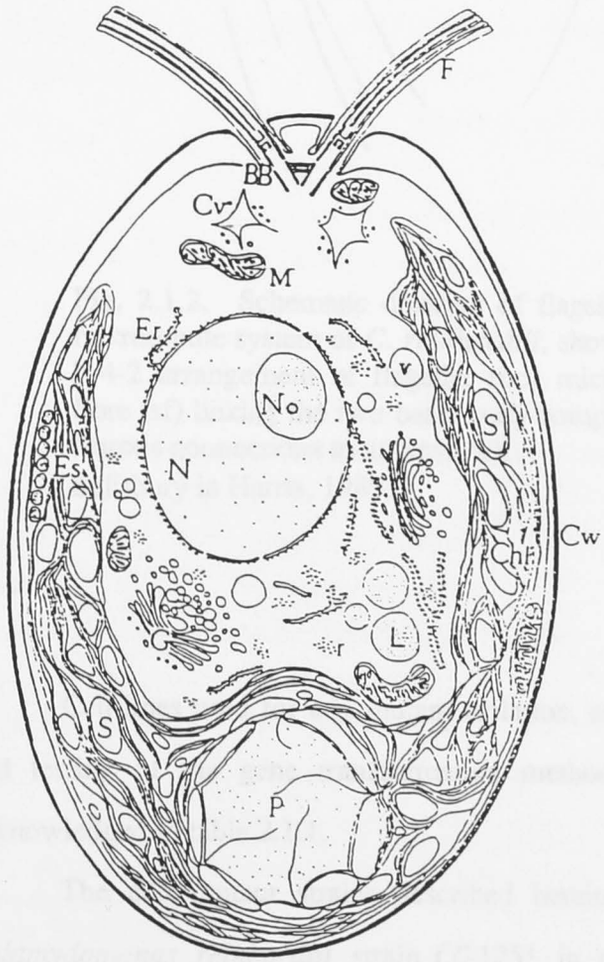


Fig. 2.1.1 A diagrammatic representation of a typical interphase cell of *C. reinhardtii*. Basal body (BB); Chloroplast (Chl); Contractile vacuole (CV); Cell wall (Cw); Endoplasmic recticulum (ER); Eye spot (Es); Flagella (F); Golgi body (G); Lipid body (L); Mitochondria (M); Nucleus (N); Nucleolus (No); Pyrenoid (P); Ribosome (r); Starch grain (S); Vacuole (V) (Adapted from Ettl;1976 and Harper, 1983 Ph.D thesis)

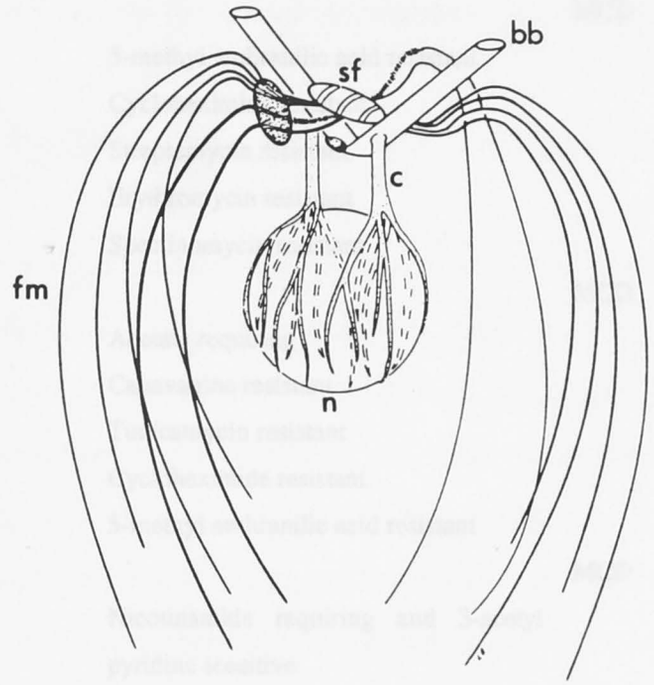


Fig. 2.1.2. Schematic drawing of flagellar roots and the cellular microtubule system of *C. reinhardtii*, showing a basal body (bb), 4-2-4-2 arrangement of flagellar root microtubules (fm), a striated fibre (sf) linking the two basal body components and the centrin (c) fibrous connections to nucleus (n). (adapted from figure by J. Salisbury in Harris, 1989).

Cell lines used for cdc mutant isolation, autolysin preparation, genetic mapping and testing of the gene transformation method were kindly provided by sources acknowledged in table 2.1.1.

The cdc mutant strains described herein were isolated from the wild type *Chlamydomonas reinhardtii* strain CC-125^{+} in our laboratory by Dr. John Harper (Harper, 1986) or by myself.

Table 2.1.1 Source of strains used

Strain	Phenotype	Source*	Provided by
CC-124⁻	Wild-type isolate	CGC	Dr. E. Harris
CC-125⁺	Wild-type isolate	CGC	Dr. E. Harris
MS I (4D)		MCD	Dr. S. Dutcher
maa8	5-methyl anthranilic acid resistant		
act2	Cycloheximide resistant		
sr1	Streptomycin resistant		
ery1	Erythromycin resistant		
Spr1	Spectinomycin resistant		
MS II		MCD	Dr. S. Dutcher
ac17	Acetate requiring		
can1	Canavanine resistant		
TUN1	Tunicamycin resistant		
act1	Cycloheximide resistant		
maa13	5-methyl anthranilic acid resistant		
MS IV (8D)		MCD	Dr. S. Dutcher
nic1	Nicotinamide requiring and 3-acetyl pyridine sensitive		
maa4	5-methyl anthranilic acid resistant		
y1⁺	Yellows in darkness		
pyr1	Pyrithiamine resistant		
MS 4 (1D)		MCD	Dr. S. Dutcher
nic13	Nicotinamide requiring and 3-acetyl pyridine sensitive		
ery3	Erythromycin resistant		
gln1	Cannot use glutamine		
CC-943 ery-2b mt⁺	Erythromycin resistant	CGC	Dr. E. Harris
CC-124⁻ agg1	Modified phototactic aggregation	MCD	Dr. E. Harris
ac-177	Acetate requiring	CGC	Dr. E Harris
CWdArg2⁻	Cell wall deficient and arginine requiring	BUCL	Dr. S. Purton

*CGC=Chlamydomonas Genetics Center, Department of Botany, Duke University, Durham, NC27706,USA. MCD=Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO80309-0347, USA. BUCL=Department of Biology, University College London, London WC1E 6BT, UK.

2.2. Methods

2.2.1 Growth media for *Chlamydomonas*

Several different media were used for various aspects of *Chlamydomonas* culture:

HSM "High Salt Medium was based on the medium of Sueoka et al (1967). It is suitable for 2 to 6 months storage of strains as stabs.

LSHSM "Low Sulphur and High Salt Medium" was based on HSM, with contains sulphur reduced to 0.07 mM and Mg^{++} held at 0.81 mM with additional $MgCl_2$ (Rollins et al., 1983). This medium has been optimally used for synchronisation and large scale culture of both wild type and cdc mutants.

TAPYPP "Tris Acetate Phosphate, Yeast extract and Proteose peptone" medium is a modified medium based on the TAP medium of Gorman and Levine (1965) supplemented with yeast extract and protein peptone. This medium has been successfully used in growing a variety of strains including wild type and cdc mutants both in liquid and on agar plates. It is particular suitable for shake cultures that can derive carbon from the acetate.

-NHSMA "Minus nitrogen, HSM Medium plus 12 mM Acetate", is a medium based on that of Van Winkle-Swift (1977). This medium does not contain a nitrogen source and is suitable for induction of gametes.

MAT Agar VW-N-Ac "Maturation medium" is based on that of Van Winkle-Swift (1977). It is suitable for maturation of *Chlamydomonas* zygospores and normally made as 4% agar plates, which provide a substratum for zygospore attachment.

The composition of these media and the stock solutions are summarised in Table 2.2.2 and Table 2.2.3 respectively

Table 2.2.1 Composition of the media

Compound	HSM	LSHSM	TAPYPP	-NHSMA	MAT Agar VW-N-Ac
NH_4Cl	9.35 mM	9.35 mM	9.35 mM	-	-
$MgSO_4$	0.81 mM	0.05 mM	0.41 mM	0.81 mM	0.081 mM
$MgCl_2.6H_2O$	-	0.76 mM	-	-	-
$CaCl_2$	0.13 mM	0.13 mM	0.13 mM	0.13 mM	0.13 mM
K_2HPO_4	8.29 mM	8.29 mM	0.63 mM	8.29 mM	8.29 mM
KH_2PO_4	5.18 mM	5.18 mM	0.39 mM	5.18 mM	5.18 mM
Tris	-	-	20.0 mM	-	-
Na-acetate	-	-	-	12.0 mM	-
Fe-EDTA	1 ml/l	1 ml/l	-	1 ml/l	1 ml/l
Glacial acetic acid	-	-	1 ml/l	-	-
Trace element solution	1 ml/l	1 ml/l	-	1 ml/l	1 ml/l
Hunter's trace element	-	-	1 ml/l	-	-
Difco yeast extract	-	-	0.04%	-	-
Difco proteose peptone	-	-	0.04%	-	-
*Difco-Bacto Agar	1.5%	1.5%	1.5%	-	4%

*Added when solid media were required. All media pH 7.0.

Table 2.2.2 Media stock additives

Compound	Trace Element Solution	Hutner's Trace Elements	Fe-EDTA Solution
H_3BO_3	1 mM	185 mM	-
$MnSO_4.4H_2O$	1 mM	7.2 mM	-
$ZnSO_4.7H_2O$	1 mM	31 mM	-
$CuSO_4.5H_2O$	10 mM	3.2 mM	-
$(NH_4)_6Mo_7O_{24}.4H_2O$	10 mM	-	-
$FeCl_3.6H_2O$	-	18 mM	-
$Na_2MoO_4.2H_2O$	-	4.1 mM	-
$CoCl_2.6H_2O$	-	0.8 mM	-
Na_2 EDTA.$2H_2O$	-	54 mM	25 mM
$FeSO_4.7H_2O$	-	-	25 mM
Conc. H_2SO_4	18.7 mM	-	-

2.2.2 Storage of *Chlamydomonas* stocks

For short term maintenance (1-2 month), cells were streaked on to TAPYPP agar plates and grown under cool white fluorescence light for 3-5 days, then transferred to low light at 21°C. Subcultures were made monthly by streaking cells from each stock onto a new TAPYPP agar plate. For maintenance of cdc mutants, and to avoid back mutations, at least 4-8 single colonies from each stock were streaked onto pairs of TAPYPP agar plates and tested at both 21°C and 33°C.

For medium term maintenance (3-6 months), all useful strains were kept as stab cultures in sterile soda-glass vials in 2.5 ml of HSM 1.5% agar. To make stabs, a loopful of cells was stabbed directly into the agar. After 2-3 days incubation in light, the vials were sealed with Nescofilm and stored in low light at room temperature.

For long term storage (more than one year), cells were cultured in LSHSM liquid medium and treated with dimethyl sulphoxide (DMSO) by adding DMSO to the culture to a final concentration of 7% (v/v). The tubes, each with 1ml of DMSO-treated cell cultures were incubated in light at 21°C for 5 hours with shaking, then transferred into a polystyrene box and slowly frozen at -80°C, then stored at that temperature. Cells were retrieved from storage at -80°C by quickly thawing the tube and immediately spreading 0.2 ml of the stock culture onto TAPYPP agar plates (2.2.1). Plates were placed in the light at 21°C for 10 days until single colonies grew to about 1-2 mm in diameter.

2.2.3. Synchronisation of the *Chlamydomonas* culture

Synchronous cultures were used in most of the experiments including those with wild type and all cdc mutants investigated. For synchronisation, precultures were prepared by inoculating a loopful of cells from stock agar plates into 250 ml conical flasks containing 50 ml of TAPYPP liquid medium and culturing at 21°C and a light intensity of 200 $\mu E\ m^{-2}\ s^{-1}$ of PAR in the range 400-700 nm for 24 to 48 hours. Synchronisation was started by transferring the preculture into 2 litres of sterile LSHSM medium (see 2.2.1), contained in 2.5 litre Roux bottles, to a cell density of $1x10^5$ cells/ml. The cultures were aerated through a tube extending to the bottom of the bottle

and terminating in a glass scinter through which sterile-filtered air enriched to 0.5% CO_2 was passed at 1 l/min. Cultures were illuminated by cool white fluorescent tubes to give a light intensity of 200. $\mu Em^{-2}\ s^{-1}$ of PAR. Alternating cycles of 14h light/10h dark (L/D) were provided and the culture was diluted to a cell density of $1x10^5$cells/ml at the end of each dark period (approximately equivalent to an O.D.680nm of 0.1). Good synchrony was achieved after 3-5 cycles as shown in Fig.2.2.1:

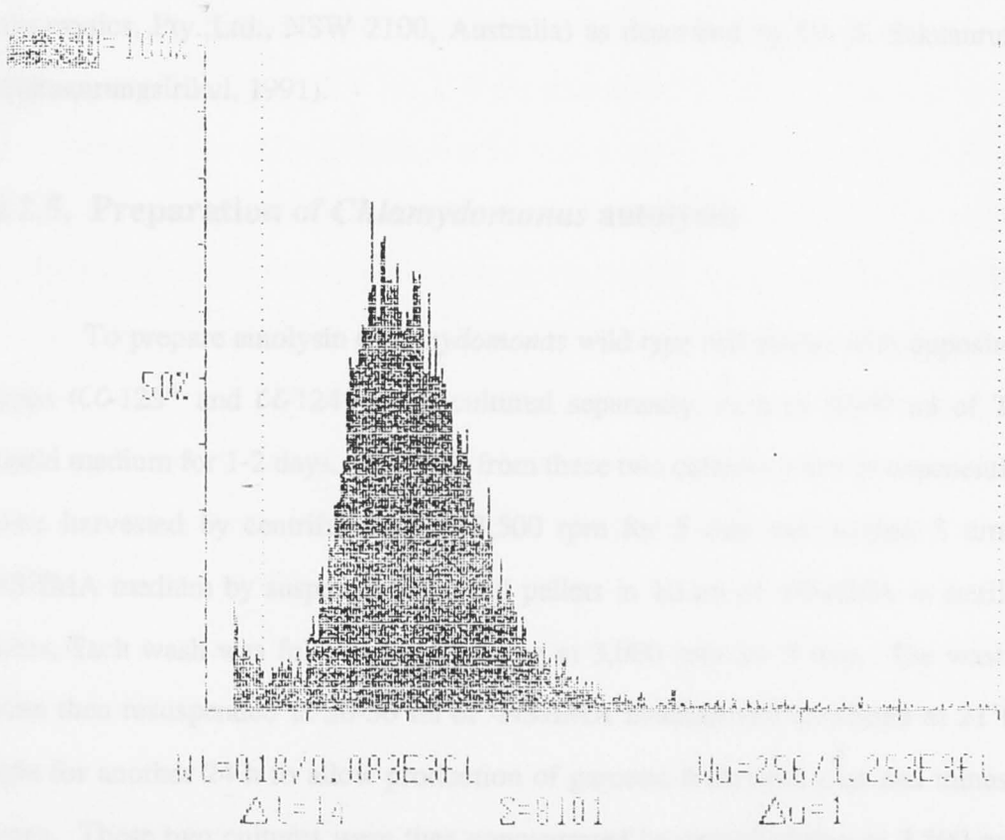


Fig. 2.2.1. Cell size frequency profile, illustrating a uniform population of *C reinhardtii* after a successful synchronisation. Cells were grown in LSHSM medium with alternating 14:10 light:dark periods and continuously bubbled with 0.5% CO_2 in air. Ordinate: percentage of population in size class; abscissa:cell volumes, with vertical dotted lines (window margins) indicating at left 83 fl (0.083 E3fl) and at right 1,253 fl (1.253 E3fl). The peak of cells, sampled in late G1 phase is centred on 330 fl. When in use the window margins (dotted lines) can be moved by keys and the position at which half of the cell number is excluded is revealed by the changing total count displayed centrally; then the size at which this occurs (the window setting) can be read off. The display represented indicates a count of 8101 cells of sizes falling between the window settings.

2.2.4. Estimation of cell number and cell size by coulter counter

To estimate cell number and size, samples from cell cultures were fixed by adding 35% formaldehyde to the culture samples to a final concentration of 1%. 0.5 ml of each fixed sample was then mixed with 24.5 ml of 0.9% sodium chloride solution made with double glass distilled water and counted on the Coulter Counter model ZM (Coulter Electronics, Pty.,Ltd., NSW 2100, Australia) as described by Dr. S. Sakuanrungsirikul (Sakuanrungsirikul, 1991).

2.2.5. Preparation of *Chlamydomonas* autolysin

To prepare autolysin *Chlamydomonas* wild type cell strains with opposite mating types (*CC*-125$^+$ and *CC*-124$^-$) were cultured separately, each in 30-50 ml of TAPYPP liquid medium for 1-2 days. The cells from these two cultures when in exponential phase were harvested by centrifugation at 3,500 rpm for 5 min and washed 3 times in -NSHMA medium by suspending the cell pellets in 10 ml of -NSHMA in sterile falcon tubes. Each wash was followed by spinning at 3,000 rpm for 5 min. The washed cells were then resuspended in 30-50 ml of -NSHMA medium and incubated at 21°C under light for another 24 h to allow production of gametes from both plus and minus mating types. These two cultures were then concentrated by centrifugation at 3,500 rpm for 5 min resuspending the cell pellet of each mating type in 15-25 ml of -NSHMA medium. The two concentrated cultures were then mixed together at the ratio of 1:1 and set in the light at 21°C for 30-60 min without agitation. After this, the mixture was centrifuged at 3,500 rpm for 10 min and the supernatant was retained and filtered to be used directly or kept at -20°C for future use. Activity was lost quickly by the frozen enzyme and fresh unfrozen enzyme was preferred.

2.2.6. Treatment of *Chlamydomonas* cell cultures with the microtubule drug oryzalin

A 10 mM oryzalin solution was prepared in DMSO and diluted into a synchronous culture at 7 h of the cell cycle to the final concentration required. The maximum oryzalin concentration, of 60 µM, therefore resulted in a final DMSO concentration of 1%, which had no detectable effect on the cells. After 24h the effect of the drug was observed by immunofluorescence microscopy. To investigate the capacity for microtubule recovery after treatment, cells which were treated with oryzalin from 7 h of the cell cycle were washed twice in oryzalin-free TAPYPP medium and resuspended in the same volume of fresh medium at 16 h, 20 h and 24 h of the cell cycle. Samples for observation of microtubule recovery were taken 1-4 h after return to oryzalin-free medium; at 20 h (for the sample washed at 16h), 24 h (for the samples washed at 20 h) and 25 h (for the sample washed at 24 h) of the cell cycle. To investigate the effect of oryzalin on $p34^{cdc2}$ kinase activity, samples were taken from both non-oryzalin treated and oryzalin treated cell cultures at 0 h, 7 h, 10 h, 12 h, 15 h, 17 h, 20 h and 24 h. Cell samples were washed 2 times in 0.025 M Tris-HCl pH 7.4 and then frozen in liquid nitrogen and kept at -80°C.

2.2.7. Staining nuclear DNA with propidium iodide (PI)

Cells in 1 ml culture samples were pelleted by centrifugation in eppendorf tubes at 3,000 rpm for 1 min and fixed in 25% ethanol for 5 min, then decolourised by dehydration in 50% ethanol for 2 min, then 80% ethanol for 5 min (at this stage, fixed cells can be kept in 80% ethanol at -20°C for several weeks to months). Fixed cells were rehydrated gradually in PBS (Phosphate Buffered Saline: 15 mM KH_2PO_4, 7.4 mM Na_2HPO_4, 163 mM NaCl, 2.7 mM KCl, 3 mM EGTA and 0.02% NaN_3) and spun to discard the supernatant. The cell pellet was then resuspended in 100 µl of 1 mg/ml RNAse solution at 37°C for 30 min. RNAse treated cells were washed by resuspension in 1 ml of 10 mM Tris pH 7.4 and spun at 3,000 rpm for 1 min. Thereafter, pelleted cells

were suspended in a small volume of the same buffer and placed onto coverslips which had been coated by drying down ethanol fixed chick erythrocytes to provide reference intensity, then stained with 25 µl of 2.5 µg/ml propidium iodide (PI) in 5 mM Tris, 1 mM EDTA pH 7.4 and 50% ethanol for 3 h at room temperature. The coverslips were placed on slides, mounted with "Antifade AF1" in Glycerol/PBS (Citifluor Ltd., London, U.K.) and sealed with nail vanish. Slides were observed on an Axioplan Universal Microscope (Zeiss, West, Germany) using a rhodamine filter.

2.2.8. Staining nuclear DNA with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI)

For staining nuclear DNA with DAPI, cells were fixed as described in 2.2.7. After rehydration, the cells were dried onto slides. About 25 µl of 0.1 µg/ml DAPI in PBS was dropped onto the slide to cover cells and stained for 5 to 10 min at room temperature. For double labelling of fluorescence conjugated antibodies together with DAPI, slides with cells that had been labelled with fluorescently tagged antibodies were incubated in 0.1 µg/ml DAPI solution for 10 to 15 min. After one wash in PBS, pH 7.4 for 5 min, the slides were briefly dried in air, mounted with "Antifade AF1" (Glycerol/PBS, Citifluor Ltd., London EC1V 0HB, U.K.) and sealed with nail vanish. A U.V. filter was used for the observation of the DAPI stained cells under an Axioplan Universal Microscope (Zeiss, West Germany).

2.2.9. Quantitative measurement of nuclear DNA by confocal fluorescence microscopy

Nuclear DNA was quantitatively measured using a method described by Dr. S. Sakuanrungsirikul (Sakuanrungsirikul, 1991). The measurements described here were to determine whether the DNA content of arrested mutant cells corresponded with that of the cells known to be in G1 phase or was doubled, indicative of G2 phase. To achieve this, cells were first stained with PI, as described above in 2.2.7. Observation and

analysis of the fluorescence samples were made on a Bio-Rad MRC-500 confocal laser scanning microscope.

Determination of the nuclear DNA levels of individual nuclei was estimated by: (1) collecting a series of measurements of fluorescence at different planes of focus, (2) measuring fluorescence intensity and area of the nuclear signal and of the surrounding cytoplasm in each plane of focus and (3) calculating the total DNA content of each three-dimensional nucleus by computing the data obtained from each plane of focus at known spacing using a computer program developed by Mr. Nicholas P. John. The relative nuclear DNA level of each mutant was sometimes evaluated as a percentage of the fluorescence of adjacent chicken red blood cells on the same coverslip. The red blood cells were used as an internal test of the consistency of staining and of measurement in individual preparations, and when freshly obtained could be used as a calibration standard to estimate the absolute weight of DNA in the *Chlamydomonas* nucleus as demonstrated earlier (Sakuanrungsirikul, 1991). About 50 cells were assayed for each sample of the cdc mutants.

2.2.10. Indirect immunofluorescence microscopy

Immunofluorescence microscopy was used to clarify the terminal phenotypes of the cdc mutants. Methods for immunofluorescence staining using different antibodies were based on the description by Harper et al. (1990a, 1990b).

The antibodies used for inspection of different structures included:

Anti-β-tubulin monoclonal antibody (Amersham, Australia, Pty.Ltd.) used for labelling was diluted to 1:500 in PBS, pH 7.4 with 3% bovine serum albumin (BSA).

Anti-acetylated tubulin antibody was kindly provided by Dr G. Piperno, the Rockefeller University, N.Y. USA. For labelling, antibody was diluted to 1:20 in PBS, pH 7.4 with 3% BSA.

Anti-centrin polyclonal antibody was a kind gift from Prof J.L. Salisbury, Department of Biochem. and Mol. Bio., Mayo Clinic, Rochester,

Minnesota, USA. The concentration of the antibody used for labelling was 1:100 in PBS, pH 7.4 containing 3% BSA.

Anti-MPM-2 monoclonal antibody was provided by Prof. P.N. Rao, Department of Medical Oncology, University of Texas, Houston, Texas USA. For labelling, the antibody was diluted to 1:800 in PBS, pH 7.4 containing 3% BSA.

Second antibodies:

FITC-sheep antimouse and FITC-rabbit antimouse antibody (Amersham) were diluted to 1:40 in PBS, pH 7.4 with 3% BSA.

The reagents used for immonofluorescence labelling include:

Phosphate buffered saline (PBS): 15 mM KH_2PO_4, 7.4 mM Na_2HPO_4, 163 mM NaCl, 2.7 mM KCl, 3 mM EGTA and 0.02% NaN_3.

Microtubule stabilising buffer (MTSB): 50 mM PIPES, 5 mM EGTA and 1 mM $MgSO_4$.

Fixation solution: 4% paraformaldehyde, 1% dimethylsulphoxide (DMSO) in MTSB.

Extraction buffer: 3% nonidet (NP-40), 5% DMSO in MTSB.

Fresh autolysin: see 2.2.5.

Poly-L-lysine solution: 1 mg/ml in water, pH adjusted to 8.5.

DAPI staining solution: 0.1 µg/ml 4',6-diamidino-2-phenylindole in PBS, pH 7.4.

β-tubulin and centrin labelling:

Samples containing approximately $1x10^6$ cells were fixed in fixation solution, described above, for 30 min. After being washed 3 times in PBS by centrifugation, the fixed cells were resuspended in fresh autolysin solution (2.2.5) for 1 h at room temperature, on a rotator, to digest the cell wall. Thereafter autolysin treated cells were washed once in TMSB and resuspended in 3% NP-40, 5% DMSO extraction buffer at room temperature for 3 h with rotation. This step was followed by 3 washes in MTSB to get rid of the extraction buffer. A more concentrated cell suspension was then made

by spinning cells at 3,000 rpm in an eppendorf tube for 1 min and resuspending the cell pellet in 100 µl of MTSB. About 5 µl of this cell suspension was spread onto poly-l-lysine coated multiwell slides and briefly dried in air. The slides were incubated in -20°C cold methanol for 10-15 min and then rehydrated by incubation in PBS (pH 7.4) for 5 min. The cells were then incubated in anti β-tubulin or anti centrin antibody solution (see above) overnight at 20°C in a high humidity chamber. After washing the slides 3 times (5 min for each time) in PBS to get rid of the unbound first antibody, FITC conjugated secondary antibody, at 1:40 in PBS containing 3% BSA, was added to the cells. Cells were incubated in second antibody at 20°C for 3 h or overnight and then washed in PBS three times. For double labelling with DAPI, the slides were incubated in 0.1 µg/ml DAPI stain solution as described in 2.2.8.

MPM-2 and acetylated tubulin labelling:

The MPM-2 proteins and the acetylated tubulin were labelled respectively by the anti MPM-2 antibody (1:800) and anti acetylated antibody (1:20). The labelling procedure was similar to that for the β-tubulin and centrin described above except that extraction in 3% NP-40 and 5% DMSO was omitted. Cells after autolysin treatment were washed once in MTSB before they were centrifuged and resuspended in 100 µl MTSB.

2.2.11. Isolation of temperature sensitive cell division cycle (cdc) mutants and suppressors of the cdc mutants

Isolation of cell division cycle (cdc) mutants:

Mutations were induced by treating wild-type cells with ethyl methane sulphonate (EMS, purchased from Sigma) and screening replica plated colonies for clones that grew at 21°C but were blocked at 33°C on TAPYPP plates. For mutagenesis, cells of wild-type *Chlamydomonas* (cc-125$^+$) were grown synchronously as described in 2.2.3. Samples (1 ml of cells for each sample) were taken in 1 h intervals beginning at 11 h in

the cell cycle and the sampled cells were stained with DAPI as described in 2.2.8 and inspected by fluorescence microscopy to determine the time when 50% of cell in the synchronous population became binucleate, indicating that many cells were in their second and final round of DNA replication. S phase cells were harvested by centrifugation at 3000 rpm for 5 minutes. The pelleted cells were resuspended in LSHSMA medium (LSHSM medium plus 12 mM Na acetate). The cell suspension was then mixed with stock filter-sterilised 400 mM EMS to obtain a final concentration of 140 mM EMS at $1x10^6$ cells /ml (Sakaunrungsirikul, 1991). The cells were shaken under continuous light at 21°C for 1 h, then washed twice with LSHSMA medium and diluted 3-5 times with LSHSMAYPP medium (LSHSMA medium plus 0.04% Difco proteose peptone and 0.04% Difco yeast extract). All medium containing EMS was mixed with 1 M NaOH to inactivate it. After 18-24 h of shaking at 21°C to allow mutagenised cells to complete their cell cycle, cells were diluted to 2500 cells/ml and spread on TAPYPP plates at 500 cells/plate. The plates were incubated at 21°C under continuous light for 6-10 days until colonies derived from single cells were evident. The technique of replica plating was used for screening temperature sensitive cdc mutants (Sakaunrungsirikul, 1991). Each pair of replica plates was screened, to detect colonies that could grow at 21°C but were unable to proliferate at 33°C, by incubation of one at 21°C and the other at 33°C for 24-48 h. After initial screening by inspection of the 33°C plate visually and under the microscope, each of the selected colonies was picked off the corresponding 21°C plate and inoculated into liquid TAPYPP medium. The liquid culture was divided and incubated at 21°C and 33°C to confirm that the cells proliferated at 22°C but arrested as big cdc cells at the non-permissive temperature of 33°C. The test in liquid medium eliminated mutants that arrest only on agar. Each isolated temperature sensitive cdc mutant was then picked off the 21°C plate and streaked on a TAPYPP plate. After growing for 5-6 days in the light at 21°C, the plate was transferred to low light as a stock for future genetic analysis.

Isolation of suppressor mutations of the cdc mutants:

Suppressor mutations were induced by treating synchronous S phase cells of cdc mutants with EMS in a final concentration of 180 mM, using the procedure described

above for the original isolation of temperature sensitive cdc mutants. After 1 h treatment with EMS, the cells were washed twice in LSHSMA and then diluted to 4 times lower than the original cell density of $1x10^6$ cells/ml with LSHSMAYPP medium. After 18-24 h shaking in the light at 21°C, the cells were spreaded on TAPYPP agar plates at 10,000 cells/plate and then incubated in the light at 33°C. Most cells died at this temperature which was restrictive for the original cdc mutation. After 10 days incubation at 33°C, the colonies that grew at 33°C were picked off the plates and streaked on new TAPYPP plates. Each colony was then backcrossed (see 2.2.12) with a wild-type cell line to test whether a back mutation, or a suppressor mutation in a separate gene, was present. The former would yield all wild-type progeny. It was hoped that some suppressor mutations might have mitotic phenotypes in the absence of the mutation that they suppressed. The isolated suppressor cell lines were then maintained in low light as stocks.

2.2.12. Backcrossing cdc mutants with the wild type

Backcrosses of the cdc mutants with wild type cell lines was performed to ensure the presence of single gene mutations in a wild type background. To achieve this, both wild-type mating types (*CC-124*$^-$ or *CC-125*$^+$) and cdc mutants (or suppressors) isolated by mutagenesis were first cultured separately in TAPYPP medium on a shaker at 21°C for 1 to 2 days with continuous illumination. When cells reached a cell density of $0.5x10^6$ cells/ml (an O D 680 of approximately 0.5), they were harvested by centrifugation (3,000 rpm for 5 minutes) and washed 3 times in -NSHMA medium (see table 2.2). The cells were resuspended in -NSHMA medium to a cell density of $0.5x10^6$/ml and shaken in the light at 21°C for one day. Equal volumes of each mutant cell culture was mixed with a wild type cell culture of the opposite mating type. The mixtures were incubated in low light at 21°C without agitation to allow cells to mate and form zygospores. From each mixture, 100 µl of cells was taken respectively at 1 h, 3 h and 6 h after mixing and spread onto a maturation agar plate lacking both nitrogen and acetate (see table 2.2.). The plates were left in light at 21°C for 24 h then transferred to a petri dish box to let the zygospores mature in total darkness for 5-6 days. This

darkness was useful for minimising zygospore growth and for obtaining offspring number of 4 rather than 8 or 16. To germinate zygospores, a block of agar containing zygospores was cut and transferred into a sterile petri dish. This block of agar was then covered with 300 µl of -NSHMA liquid medium. Individual matured zygospores from different crosses were picked up from the agar block under a microscope using a fine capillary and transferred onto a germination plate (see table 2.2.). One zygospore was delivered onto one of the 1 cm squares, marked as shown in Fig. 2.2.2., by blowing gently into a tube attached to the capillary. One zygospore from each 0.5 cm (or 1cm) square was moved down and located at the extreme top of the rectangle marked in Fig. 2.2.2. using a "moving rod" on a micro-manipulator (Leitz Wetzlar). The moving rod comprised a finely drawn glass rod ending in a knob. Germination plates with individual zygospores located in the top end of each rectangles were incubated in light at 21°C for 18-24 h to allow the diploid zygospores to germinate meiotically and release tetrads of haploid offspring. when a zygospore released its progeny, the four daughter cells were moved apart by 3-4 mm before any of them completed their first mitotic division. Separated tetrads were then left in continuous light at 21°C for one week to form small colonies. The colonies were streaked onto agar before they could merge and then screened at both 21°C and 33°C to determine the ratio of cdc:wild-type for genetic analysis. Good cdc progeny from each cross were kept as higher F number cell lines, or for further backcrosses.

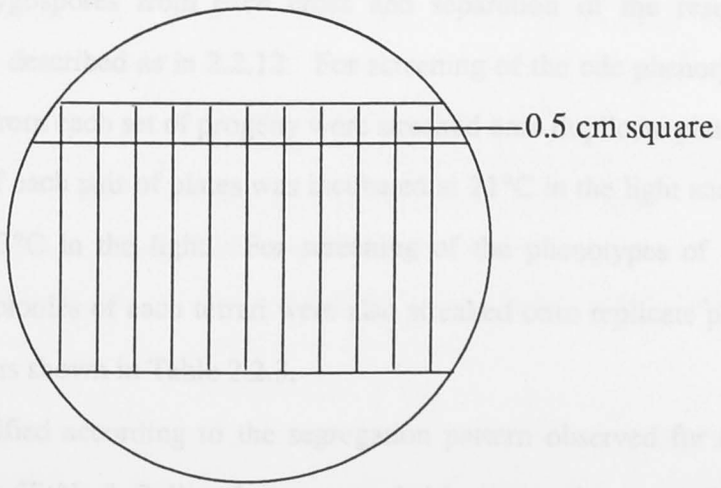


Fig. 2.2.2. Germination plate for separation of progeny from zygospores.

2.2.13. Genetic mapping of cdc mutations

Genetic mapping of cdc mutations was carried out by tetrad analysis, after crossing the cdc mutants with mapping strains that carry genetic markers for each of the 17 linkage groups reported to date in *C. reinhardtii*.

Previously mapped marker genes were selected to include at least one from each of the 17 linkage groups. In the case of the larger linkage groups more than one marker gene was used so that a mapping distance of 50% recombination units from both sides of the markers could cover most of the mapped loci reported up to date. The chosen markers, their phenotypes and the conditions for screening them are summarised in Table 2.2.3. The chromosomal locations of these markers are indicated later in Fig. 3.4.9.

For crosses of the cdc mutants and mapping strains, cdc mutant cells and different mapping strains were cultured separately in 5-10 ml of TAPYPP liquid medium at 21°C for 24-48 h with continuous illumination until growth to late exponential phase was visible. The cells of each culture were then washed 3 times in -NSHMA medium and resuspend in -NSHMA to a cell density of about 0.5×10^6/ml. The cell suspensions were shaken in light at 21°C for another 24 h. Then 0.5 ml of each cdc cell culture was mixed with 0.5 ml of cells from each mapping strain and incubated in low light without agitation to mate and form zygospores as in 2.2.12.

Germination of zygospores from each cross and separation of the resulting tetrads were performed as described as in 2.2.12. For screening of the cdc phenotypes, the four colonies derived from each set of progeny were streaked onto duplicate plates of TAPYPP medium. One of each pair of plates was incubated at 21°C in the light and the other was incubated at 33°C in the light. For screening of the phenotypes of each genetic marker, the four colonies of each tetrad were also streaked onto replicate plates providing the test conditions shown in Table 2.2.3.

Tetrads were classified according to the segregation pattern observed for each pair of markers in the cross (Table 2. 2 4) and were recorded in terms of parental ditype (PD), nonparental ditype (NPD) and tetratype (T). Linkage or nonlinkage was

determined by the ratio of PD to NPD to T, as described by Harris (1989). The segregation of these three types of tetrads is shown in Table 2.2.5, and some sample data from typical crosses of *C. reinhardtii* in Table 2.2.6.

Table 2.2.3 Chosen markers, phenotypes and conditions for screening for genetic mapping

Marker	Phenotype	Conditions for screening
ery3	Erythromycin resistant	100 µg/ml erythromycin in selecting medium
act1	Cycloheximide resistant	15 µg/ml cycloheximide to selecting medium
ac17	Acetate requiring	Growth only when acetate is added to the medium (0.1% sodium acetate)
pyr1	Pyrithiamine resistant	1µg/ml pyrithiamine in selecting medium
pf1	Paralysed flagella	Observe living cells in liquid medium by Nomarski microscopy
maa8	5-methyl anthranilic acid resistant	175 µg/ml 5-methyl anthranilic acid in medium, wrap plates with two layers of paper towel
act2	Cycloheximide resistant	15 µg/ml cycloheximide in selecting medium
can1	Canavanine resistant	300-500 µg/ml canavanine in selecting medium
TUN1	Tunicamycin resistant	5 µg/ml tunicamycin in selecting medium
sr1	Streptomycin resistant	75 µg/ml streptomycin in selecting medium
nic13	Nicotinamide requiring and 3-acetyl pyridine sensitive	Lethal on medium with 7.5 µg/ml 3-acetyl pyridine and without nicotinamide
ery1	Erythromycin resistant	100 µg/ml erythromycin in selecting medium
gln1	Can not use glutamine	No growth on medium with glutamine as sole N source
agg1	Modified phototatic aggregation	Cells form pellet in bottom of the tube when exposed to overhead light
maa4	5-methyl anthranilic acid resistant	175 µg/ml 5-methyl anthranilic acid in medium, wrap plates with two layers of paper towel
ery2	Erythromycin resistant	100 µg/ml erythromycin in selecting medium
nic1	Nicotinamide requiring and 3-acetyl pyridine sensitive	Lethal on medium with 7.5 µg/ml 3-acetyl pyridine and without nicotinamide
y1	Yellows in darkness	Incubate plates in dark
spr1	Spectinomycin resistant	70 µg/ml spectinomycin in selecting medium
maa9	5-methyl anthranilic acid resistant	175 µg/ml 5-methyl anthranilic acid in medium, wrap plates with two layers of paper towel

Table 2.2.4 Identification of parental and recombinant tetrad type

Sample cross: *met-1+mt*$^+$ x *+nic-13 mt*$^-$		
Parental ditype (PD)	Nonparental ditype (NPD)	Tetratype (T)
met-1 +	*met-1 nic*	*met-1* +
met-1 +	*met-1 nic*	*met-1 nic*
+ *nic*	+ +	+ *nic*
+ *nic*	+ +	+ +

Table 2.2.5 Generation of PD, NPD, and T tetrads by crossing over*

Two loci, linked		Two loci, unlinked	
Crossovers	Tetrad type	Crossovers between loci and centromeres	Tetrad types
None	PD	None	1 PD : 1 NPD
Single	T	One locus and its centromere	T
Double, two-strand	PD	Both loci and their centromeres	1 PD : 2 T : 1 NPD
Double, three-strand	T		
Double, four-strand	NPD		

* Adapted from Perkins (1949)

Table 2.2.6 Sample tetrad data from *Chlamydomonas* crosses*

Relationship of loci	Tetrad distribution	Ratio PD : NPD : T	Map Distance
Closely linked loci	NPD=0; PD>T		
ac-1 x *pf-12* (II)		77:0:10	5.7
ac-30 x *pf-14* (VI)		78:0:12	6.7
Distantly linked loci, same side of centromere	PD>NPD; T>PD; T>4NPD		
act-2 x *pf-14* (VI)		23:0:34	29.8
msr-1 x *arg-2* (I)		22 :1:43	34.1
Distantly linked loci, opposite side of centromere	PD>NPD; T>NPD		
ac-15 x *sr-1* (IX)		65:1:43	20.6
thi-9 x *nic-2* (II)		6:4:36	47.8
act-2 x *thi-10* (VI)		4:5:47	50.9
Unlinked, both loci close to centromeres	T<PD or NPD; PD and NPD about equal		
pf-27 (XII) x *ac-46* (XVI)		12:14:0	-
ac-17 (III) x *pyr-1* (IV)		36:33:14	-
ac-17 (III) x *pf-2* (XI)		42:34:14	-
Unlinked, both loci distant from centromeres	PD and NPD equal, but T>PD		
act-2 (VI) x *nr-1* (VIII)		22:29:101	-
act-2 (VI) x *msr-1* (I)		18:16:78	-

* Linkage group given in parentheses. Adapted from Harris (1989).

Map distances were calculated as percentage of recombination according to the equation (Harris, 1989):

Percentage of Recombination (%) = (NPD+0.5T)/(PD+NPD+T)

PD = parental ditype

NPD = nonparental ditype

T = tetratype

2.2.14. $p13^{suc1}$ overexpression and purification

Transformant cells of BL21 (DE3) harbouring plasmids with the $p13^{suc1}$ gene of S. pombe derived by the T7-bacteriophage promoter (Studier and Moffat,1986) were used for overexpression of $p13^{suc1}$.

Cells from the BL21 (DE3) frozen stock were inoculated into 400 ml of LB medium containing 100µg/ml Ampicillin (Amp) and incubated in a shaking incubator overnight at 37°C as a starter culture. The starter culture was then transferred into 39.5 litres of LB/Amp medium and grown at 37°C until cells reached exponential stage. 3.8 g IPTG (in 25 ml) was added to the culture to allow the $p13^{suc1}$ to be expressed for the next 3 h. Thereafter, the cells were harvested using a Sharples continuous flow centrifuge.

Harvested cells were washed in 20 mM Tris pH 7.5 by suspending cells in the buffer and then recovering the cells by centrifugation; the cells were kept at 4°C. Cells were then resuspended in an equal weight of 20 mM Tris buffer pH 7.5 and stored on ice. After adding the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) to the cell suspension to a final concentration of 0.0174 g per 100 ml, the cells were broken open in a YEDA continuous flow cell smasher.

A procedure based on that of Labbe et al.(1989) was used to precipitate the overexpressed $p13^{suc1}$ protein from smashate. To the cold smashate, 1/10 volume of 500 mM Tris, 20 mM EDTA pH 8.0 was added. Solid ammonium sulphate was added to the smashate to a final concentration of 30%. The smashate was placed in ice for 30 minutes then centrifuged at 10,000 *g* for 10 minutes in 250 ml centrifuge bottles. The supernatant was retained and additional ammonium sulphate was added to reach a final concentration of 50%. It was then stored on ice for another 30 minutes to precipitate the $p13^{suc1}$ protein before centrifugation at 10,000 g for 10 minutes. The pellets were dissolved in 20 ml of 50 mM Tris, pH 7.5 and dialysed against 2 litres of 50 mM Tris-HCl pH 7.5, 0.05% azide in 3,500 MW cut-off dialysis tubing at 4°C for 24 h with 3 changes of the dialysis buffer.

Dialysed protein was purified by chromatography. Firstly, 1/3 of the dialysed protein precipitated from the 40 litres culture was loaded onto an equilibrited Sepharose S-200 gel filtration column (50 x 9000 mm) and collected as 150 drops per fraction at a elution speed of 85 ml/h. It was found that $p13^{suc1}$ appeared in the second big OD peak at about twice the V_0 (first big peak) volume. Location of the protein was confirmed by electrophoresis on 15% acrylamide SDS gels of 30 µl samples taken from each second fraction from the region believed to contain $p13^{suc1}$. Samples were mixed with equal volumes of Sx2 sample buffer (0.125 M Tris pH 6.8, 4% SDS, 2% glycerol, 2% mercaptoethanol and 0.002% bromophenol blue), boiled for 3 minutes and 20 µl loaded per well. All fractions containing abundant $p13^{suc1}$ protein were pooled and made to 0.85 M ammonium sulphate by adding solid ammonium sulphate. This was then loaded onto a hydrophobic chromatography phenyl sepharose 4B (Pharmacia) column (10 x 140 mm) that had been equilibrited with column buffer (0.85 M ammonium sulphate in 50 mM Tris pH 7.5). Unbound proteins were washed from the column by passing through the column one bed volume of column buffer. The bound $p13^{suc1}$ protein was then eluted with 100 ml of a declining gradient of ammonium sulphate 0.85 M to 0 in 50 mM Tris pH 7.5 and collected at 2.5 ml per fraction. The fractions that contain the purified $p13^{suc1}$ protein confirmed by electrophoresis were pooled. The purified $p13^{suc1}$ was then concentrated by precipitation with 80% ammonium sulphate and dialysed against 2 litres of 25 mM Hepes, 10 mM $MgCl_2$ and 1 mM dithiothrietol (DTT) pH 7.4 at 4°C overnight with a change of the same buffer. The dialysed $p13^{suc1}$ protein was aliquoted at a concentration of 1 mg/ml and stored at -80°C.

2.2.15. Polyacrylamide gel electrophoresis and Western blot procedues

Samples of mutant or wild type cell lines were washed in 25 mM Tris HCl pH 7.4 5 mM $MgCl_2$, pelleted cells were suspended in an equal volume of this buffer then frozen dropwise in liquid nitrogen and stored at -80°C. The frozen cells were then ground in liquid nitrogen with a cooled mortar and pestle. To prepare cell extracts, 0.1g of ground

cells of each sample was mixed with 300 µl of cold RIPA buffer (see below) and vortexed twice for 20 seconds each time, with 1 min in ice between vortexing. After centrifugation at 11,000 *g* for 5 minutes at 4°C, the supernatant was retained and mixed with an equal volume of Sx2 sample buffer (see 2.2.14) and boiled for 3 minutes. The boiled samples could be stored at -80°C prior to electrophoresis.

For electrophoresis, equal protein loading was achieved by Coomassie brilliant blue protein assay based on the method described by Spector (1978). For each sample, 50 µg of total protein was loaded onto a 15% acrylamide SDS gel and run in 25 mA/gel for 5-6 h, then electroblotted onto 0.45 µm nitrocellulose (0.1 µm nitrocellulose for $p13^{suc1}$ blotting) which was then incubated in 0.05% skim milk protein in TBS (10 mM Tris and 0.15 M NaCl, pH 7.4) buffer to block for 1 hour. The blocked nitrocellulose was washed 3 times (10 minutes for each time) in TBS buffer containing 0.05% Tween-20, before probing with primary antibodies. For detection of EGVPSTAIREISLLKE-containing $p34^{cdc2}$ like protein, a primary antibody raised against the 16 amino acid PSTAIR sequence was used in a final concentration of 1:500 in TBS pH 7.4, 1% BSA, 0.05% Tween 20 and 0.05% azide. For $p13^{suc1}$ and $p56^{cdc13}$ like protein detection, an antibody against $p13^{suc1}$ from *S. pombe* in a conc. of 1:1000 (in TBS pH 7.4, 1% BSA, 0.05% Tween 20 and 0.05% azide) and an antibody against $p56^{cdc13}$ from *S. pombe* in a conc. of 1:1000 (in TBS pH 7.4, 1% BSA 0.05% Tween 20 and 0.05% azide) were used respectively. The incubation with primary antibodies was for 1 h at room temperature or overnight at room temperature. Second antibodies were radioactive or enzyme conjugated as appropriate and will be detailed in accompanying methods.

RIPA buffer contained 20 mM Tris pH 7.4, 5 mM EDTA, 100 mM NaCl, 0.1% Tween 20, 1 mM dithiothrietol (DTT), 10 µM pepstatin A, 10 µM leupeptin, 1 mM Na fluoride, 1 mM EGTA, 1 mM pyrophosphate, it was kept at -80°C as stock solution, and just before use the following further additions were made: 12 mM β-glycerophosphate, 1 mM Na ortho vanadate and 200 µM PMSF.

2.2.16. $p34^{cdc2}$ and $p13^{suc1}$ protein level estimations

For estimation of $p34^{cdc2}$ and $p13^{suc1}$ protein, the blocked and washed nitrocellulose carrying blotted proteins was incubated in a solution containing polyclonal antibody against PSTAIR peptide at a concentration of 1:500 in TBS pH 7.4, 1% BSA, 0.05% Tween 20 and 0.05% azide for 1 hour at room temperature or overnight at 4°C. The nitrocellulose was washed 2 times in TBS 0.05% Tween 20 pH 7.4 and once in TBS 0.2% Tween 20 pH 7.4, then incubated in the second antibody solution, anti-rabbit ^{125}I-labelled F(AB')2 fragment (from donkey, Amersham) at a final concentration of 0.5 µCi/ml in TBS containing 1% BSA, 0.05% Tween and 0.05% azide, for 1h at room temperature with gentle shaking. The blot was washed 3 to 4 times (10 minutes each time) in TBS 0.2% Tween 20, and then dried in air. Finally the dried nitrocellulose was taped onto a sheet of Whatman 3MM chromatography paper, and wrapped with Vitafilm plastic wrap and exposed in a PhosphorImager cassette and quantitatively analysed by ImageQuant software Version 3.0 (Molecular Dynamics, 240 Santa Ana Court, Sunnyvale, CA 94086 USA).

2.2.17. Detection of $p56^{cdc13}$ and mitosis-specific phosphoproteins by Western blotting

For detection of the $p56^{cdc13}$-like protein in *Chlamydomonas* by Western blotting, the procedure used was similar to that for $p34^{cdc2}$ except that (1) the primary antibody was anti *S. pombe* $p56^{cdc13}$ polyclonal antibody* at a dilution of 1:1000 in TBS pH 7.4, 1% BSA and 0.05% Tween 20 and (2) the secondary antibody used for $p56^{cdc13}$ was goat anti rabbit alkaline phosphatase conjugate (TAGO, Inc., CA) diluted 1:4000 in TBS pH 7.4, 1% BSA and 0.05% Tween 20.

Mitosis-specific phosphoproteins were detected on Western blots by probing with MPM-2 antibody at 1:800 dilution in TBS, 1% BSA and 0.05% Tween 20. The basic procedure was as described in 2.2.15 and the secondary antibody was goat anti mouse

alkaline phosphatase conjugated antibody (TAGO, Inc., CA) at 1:4000 dilution in TBS pH 7.4, 1% BSA and 0.05% Tween 20.

After incubation in alkaline phosphatase conjugated antibody, the blots were washed twice (10 min each time) in TBS pH 7.4, 0.2% Tween 20, once in TBS pH 7.4 and then once (for 5 min) in alkaline phosphatase substrate buffer (0.1 M Tris, 0.1 M NaCl and 50 mM $MgCl_2.6H_2O$, pH 9.5). The blots were then incubated in alkaline phosphatase substrate solution (see below) until the reaction at positive bands was clear. The nitrocellulose was immediately removed into H_2O to stop the reaction.

Alkaline phosphatase substrate solution was freshly prepared by mixing 5 ml of alkaline phosphatase buffer with 22 µl of nitroblue tetrazolium chloride (NBT) stock solution (100 mg in 1.3 ml of 70% dimethylformide) and 16 µl of 5-bromo-4-chloro-3-indolyphosphate p-toluidine salt (BCIP) stock solution (100 mg in 2 ml of 100% dimethylformamide).

*(Moreno et al., 1989b)

2.2.18. Coupling $p13^{suc1}$ to CNBr sepharose 4B

1.5 g of freeze dried CNBr-Sepharose 4B (Pharmacia, supplied by Sigma) was swollen in 100 ml of 1 mM HCl for 15 minutes and filtered in a Millipore "G3" filter unit. The swollen Sepharose was washed with 100 ml of 1 mM HCl and then 100 ml of coupling buffer (0.1 M Na-PO_4, pH 7.5). The gel was resuspended in an equal volume of coupling buffer and transferred into a 10 ml Falcon tube. Purified $p13^{suc1}$ protein, that was previously dialysed against the coupling buffer overnight at 4°C (from which 50 µl of $p13^{suc1}$ solution was retained as a "before coupling sample"), was added to the CNBr-Sepharose to give 8 mg $p13^{suc1}$ protein per 1 ml of swollen Sepharose beads. The Sepharose and $p13^{suc1}$ mixture was rotated at 4°C overnight or at room temperature for 2 h on a rotator to let $p13^{suc1}$ become covalently linked to the Sepharose beads. The gel was then recovered by gentle centrifugation at 1,800 rpm for 2 min (50 µl of supernatant was retained as an "after coupling sample"). The recovered Sepharose beads were washed twice by resuspension and centrifugation in 10 ml of 0.5 M Na-PO_4 pH 7.5, once in 10 ml of 1 M NaCl 50 mM Na-PO_4 pH 7.5 and finally

suspended in 10 ml of 1 M ethanolamine, pH 8.0 at room temperature for 1 h on a rotator to block any unreacted groups. The blocked Sepharose beads were washed repeatedly 3 times by suspending the beads alternatively in 10 ml of 0.1 M sodium acetate buffer, pH 4.0, 0.5 M Na-PO_4 then in 10 ml of coupling buffer. After washing the beads once in PBS pH 7.5, the $p13^{suc1}$ coupled CNBr-Sepharose beads were suspended in an equal volume of PBS containing 0.01% merthiolate and kept at 4°C. To check the coupling efficiency, the retained "before coupling sample" and "after coupling sample" were electrophoresed on 15% SDS-PAGE. Before use the beads were washed twice in NDE buffer (see 2.2.19 below) and resuspended in an equal volume of NDE buffer. The preparation was then used for affinity purification of the $p34^{cdc2}$ kinase from cell extracts.

2.2.19. Affinity purification of $p34^{cdc2}$ kinase (MPF) from *Chlamydomonas*

Buffer solutions used:

a). NDE buffer (pH 7.4):

Hepes pH 7.4	20 mM
NaCl	100 mM
Dithiothreitol	15 mM
Leupeptin	3 µg/ml
PMSF	0.5 mM
EGTA	20 mM
β-glycerophosphate	80 mM
Vanadate	1 mM
p-nitrophenylphosphate	30 mM
NaF	50 mM
$MgCl_2$	15 mM
Ammonium molybdate	0.2 mM

b). HDW buffer (pH 7.4):

EDTA pH 7.2	2 mM
NaCl	150 mM
Leupeptin	5 µg/ml
PMSF	0.5 mM
β-glycerophosphate	0.1 mM
NaF	50 mM
Na-PO_4 buffer pH 7.0	10 mM
NP-40	0.2%

c). PBS-Mg-DTT buffer (pH 7.2):

PBS pH 7.4	see 2.10.
$MgCl_2$	10 mM
Dithiothrietol	1 mM

The whole procedure for the purification was performed at 4°C. To start, 0.05 g of cells ground in liquid nitrogen was mixed with 150 µl ice cold NDE extraction buffer (see above) and mixed twice by vortexing for 20 seconds each time. After centrifugation

at 11,000 *g* for 5 min at 4°C, 120 µl of supernatant was transferred into a new eppendorf tube to which 40 µl of uncoupled CNBr Sepharose bead suspension that had been equilibrated in NDE buffer was added. The mixture was rotated at 4°C for 1 h and then spun at 11,000 *g* for 5 min. The supernatant was then placed in a new tube. The supernatant was mixed with 40 µl of $p13^{suc1}$ coupled Sepharose beads and rotated at 4° C for 1 h to allow the $p34^{cdc2}$ kinase to bind to the $p13^{suc1}$ beads. The beads were recovered by centrifugation at 7,000 rpm for 3 min at 4°C followed by 2 washes in HDW buffer (see above) and one wash in PBS-Mg-DTT buffer (see above) to remove the unbound proteins. After spinning at 7,000 rpm at 4°C for 3 min and removing the supernatant, 50 µl free $p13^{suc1}$ solution at a concentration of 0.5 mg/ml that had been dialysed against 25 mM Hepes, 10 mM $MgCl_2$ and 1 mM DTT, pH 7.4 was added to the beads and rotated gently at 4°C for 10 min to elute the bound $p34^{cdc2}$ kinase (MPF) from the Sepharose beads. The purified $p34^{cdc2}$ kinase (MPF) was recovered by retaining the supernatant after centrifugation at 13,000 rpm for 5 min at 4°C.

2.2.20. $p34^{cdc2}$ kinase activity assay

$p34^{cdc2}$ kinase activity was assayed using H1 histone as the substrate. The "assay mixture" was prepared freshly to contain when mixed with 0.4 volume of enzyme, the following final concentration: 25 mM β-glycerophosphate, 10 mM $MgCl_2$, 1 mM dithiothrietol (DTT), 25 mM Hepes pH 7.3, 160 µM histone H1, 10 mM EGTA and 5µ Ci/ml ^{32}p labelled γ-ATP (Amersham).

To perform the assay, 30 µl of "assay mixture" was added to each eppendorf tube and prewarmed by incubating the tube in a 37°C water bath for 5 min. The reaction was started by adding to the "assay mixture" 20 µl of affinity purified $p34^{cdc2}$ kinase (MPF). A blank no-enzyme control was made by mixing 20 µl of distilled water and 30 µl of "assay mixture". After 5 min incubation, each of the reactions was stopped by removing the assay tube to an ice bucket and immediately taking a 20 µl sample and then spotting onto a 1x2 cm p81 phosphocellulose strip. The spotted P81 strip was left to dry in air for 30 seconds to allow the peptide to bind to the strip before it was transferred into 200

ml of 0.75 mM phosphoric acid solution. The strips were rinsed 3 times in 200 ml of 0.75 mM phosphoric acid solution for 5 min each time and lifted out with clean forceps and each placed into an eppendorf tube after quickly blotting onto tissue so that the amount of surface water on each strip was consistently low. 1.4 ml of scintillation cocktail (Packard " Emulsifier safe") was added, the tube was then inverted several times to mix the scintillant and placed into a scintillation vial and counted in a Beckman scintillation counter.

Labelled histone in the remaining 30 µl of reaction mixture was separated by electrophoresis after mixing with 30 µl of Sx2 SDS sample buffer and boiling for 3 min. The boiled sample was kept in -20°C for later electrophoresis or was directly loaded onto a 12% SDS-PAGE at 50 µl/ well. After electrophoresis, the gel was stained in 0.2% Coomassie solution (0.2 Coomassie brilliant blue G250, 40% methanol and 10% glacial acetic acid) overnight at room temperature and destained in 0.5% methanol and 0.75% acetic acid to check for resolution of the histone, which usually ran as a pair of bands close to the 30 kDa marker. The gel was then incubated in 3% glycerol, 40% methanol, 10% acetic acid for 3 h followed by incubation in 3% glycerol in H_2O for 10 min before it was dried on a Bio-Rad gel dryer. The dried gel was wrapped in Vitafilm plastic wrap and exposed in a PhosphorImager cassette for 24 h and then the resulting image analysed quantitatively on a PhosphorImager by standard procedure.

SECTION 3. RESULTS

Subsection 3.1. Determination of terminal phenotypes of cdc mutants

3.1.1. Introduction

The events of the cell cycle must occur in a particular order if successful completion of division is to occur. In genetically normal cells a failure to complete core events in the DNA replication nuclear division sequence (DNA-division sequence, Mitchison, 1971) results in a block to progress through later events in the sequence and cell cycle arrest results (Enoch and Nurse, 1990). The study of the mechanisms that ensure orderly progression through the control points of the cell cycle is one of the most active areas in cell cycle research. Two strategies for investigation have been widely used. One strategy is to use specific inhibitors to establish the role of particular gene products or reactions in the cell cycle. However, this is limited by two factors: (1) there are few inhibitors available compared with the number of cell cycle events and (2) the inhibitors for particular gene products often have side effects that may complicate the interpretation of their effects. An alternative strategy is to study mutant cells that are defective in specific stages of the cell cycle. This provides information not only about the range of functions in which a particular gene product participates, but also, in cell types amenable to genetic transformation, a powerful means to clone cell cycle genes that are identified by cdc mutations. As major "work-horses", *Saccharomyces cerevisiae* (budding yeast) and *Schizosaccharomyces pombe* (fission yeast) have made the most remarkable contribution to cell cycle research by providing an invaluable array of mutants, which have been studied by classical and molecular genetics to identify key cell cycle genes and their products. These mutants are mostly temperature-sensitive and arrest at specific points in the cell cycle (Pringle and Hartwell, 1981). Mutations affecting particular steps of the cell cycle have also been isolated in some other organisms such as the mycelial fungus *Aspergillus nidulans* (Morris, 1976a; 1976b;

1990; 1992; Oakley and Morris, 1983), the ciliated protozoan *Tetrahymena thermophila* (Frankel et al., 1980), the fruit fly *Drosophila melanogaster* (Baker et al., 1978) and the green alga *Chlamydomonas reinhardtii* (Howell at al., 1977).

Under restrictive conditions, cells of conditional cell cycle mutants cease normal development at the point in the cell cycle at which the defective gene products would normally function. Arrested cells of cdc mutants usually hold particular morphological or biochemical characteristics that have been caused by the mutation. This set of characteristics is named the "terminal phenotype" of the mutants. By analysing the terminal phenotype, it has been possible to identify where in the cell cycle a particular cdc gene acts (Pringle and Hartwell, 1981). Moreover, rescue of the mutant by genetic complementation with a genomic DNA library from the wild type has enabled particular cdc genes to be isolated and sequenced (Wheals, 1987).

The earlier attempt to isolate conditional cdc mutants from *Chlamydomonas* by Howell and Naliboff (1973) did not include an investigation of their terminal phenotype, which would have more clearly established whether they were true cdc mutations rather than mutations that blocked growth. However, a collection of *Chlamydomonas* mutants conditionally defective in cell division has been isolated in our laboratory. These mutants are mostly temperature-sensitive and have been maintained with careful selection against back mutation in each subculture. Such selection has not been carried out with the mutants isolated by Howell and Naliboff, therefore ours may now represent the only genuine plant cell division cycle mutants available.

The five mutants investigated in the present analysis were chosen from 51 temperature-sensitive cdc mutants isolated in our laboratory. These mutants were obtained in *C. reinhardtii CC-125*$^{+}$ by mutagenesis during S phase in synchronous cultures with ethyl methanesulphonate (EMS). Replicate mutagenised cultures were used to obtain independently isolated mutations. This subsection of the thesis will focus on attempts to determine the stages of the cell cycle in which the five mutants became blocked when they were grown at restrictive temperature.

3.1.2. Results

3.1.2.1. Effects of mutations on cell division or growth

A common characteristic of the five mutants under investigation is that when they were grown at the permissive temperature of 21°C they could go through their cell cycle normally but when grown at the restrictive temperature of 33°C they became blocked in the cell cycle, arresting as big spherical cells. This phenotype indicates that all these mutants were blocked in cell division rather than in growth and in synchronous cultures of the five mutants growth in cell mass, indicated by culture turbidity and cell volume kept increasing steadily even at restrictive temperature (Fig. 3.1.1). At 21°C, cells in the synchronous cultures of all the five mutants were able to complete cell division and produce daughter cells, which led to a sharp decrease in mean cell size at about 20 h. However, when cells were grown at 33°C cell division did not occur, therefore mean cell size reached a high level (Fig. 3.1.1). All these results confirmed that the cells, when incubated at restrictive temperature, were arrested in cell division but not in growth. Therefore they are all true cell division cycle (cdc) mutants.

3.1.2.2. Use of backcross to obtain a single mutated gene and wild type genetic background in cells with cdc phenotype

It is necessary to test how many genes are responsible for the cdc arrest of each mutant. If a cdc phenotype is caused by more than one gene, it is then necessary to separate the contributing mutations by further backcrosses. Phenotypes caused by single gene mutations allow investigation into functions of the normal gene and study of what subsequent events are dependent on completion of the blocked event. To obtain single mutations in a wild type background, each mutant was backcrossed with a wild type cell line, and tetrad analysis was carried out after each cross. Repeated backcrossing is valuable because each generation (F number) dilutes the concentration of other possible

modified genes that have been inherited from the original mutagenised cell in which the cdc mutation was isolated. The highest backcross F number and the phenotype distribution in tetrad progeny from the last crosses of the five mutants were shown in Table 3.1.1. Data from tetrad analysis of each backcross revealed a ratio of 2:2 (wild type : cdc phenotype), indicating that each of the five mutants identified a single modified gene encoding a protein that was necessary for completion of the cell division cycle.

Table 3.1.1. Data from backcrosses to obtain single gene mutations in wild type genetic backgrounds

Mutant	Fn*	Ratio of cdc:wild type in progeny of single zygospores	Number of zygospores scored	Number of mutant genes responsible for phenotype
cdG1-1	F_5	2:2	24	1
cdM-1	F_9	2:2	37	1
cdM-2	F_6	2:2	29	1
met-1	F_6	2:2	45	1
cdCK-1	F_3	2:2	20	1

*Fn=generation number.

3.1.2.3. Analysis of nuclear DNA levels in arrested cells

For determining the phase of cell cycle arrest, it is necessary to measure nuclear DNA content in arrested cells. A widely used method for DNA estimation is to measure the deoxyribose present in DNA that has been extracted from cells and reacted with chemicals such as diphenylamine (Hopkins et al., 1972). This method has been successfully used in determination of the time of DNA replication in synchronous cultures of *Chlamydomonas* since the increase in DNA is often 4 to 8 fold. However, the method cannot eliminate contributions from other cell constituents such as the mitochondria and chloroplasts or interference from other chemicals. This interference is

especially troublesome in the case of cell division cycle mutants since arrested cells often attain big sizes and also in some arrested cells the chloroplast and mitochondrial DNA may become enormously increased. A method has therefore been developed in our laboratory (see Sakuanrungsirikul, 1991) to measure DNA directly in nuclei of whole fixed cells by using a DNA-intercalating fluorescent nuclear stain, propidium iodide (PI). The fluorescence intensity from the stained nuclear DNA was assayed in a series of optical sections at different planes of focus, in a confocal microscope. Only the signals from the nuclear DNA were collected to establish the true nuclear states of each mutant. About 50 cells were assayed for each sample of the mutants at both permissive and non-permissive temperatures. The nuclear DNA levels were calculated in whole nuclei by three dimensional reconstruction (Sakuanrungsirikul, 1991). Analysis of DNA level in arrested cells using this method is extremely slow, but it was absolutely necessary.

Data from the assay of nuclear DNA content in the mutant *cdG1-1* showed that after the cells became arrested at 33°C, the content of the nuclear DNA retained a similar distribution to that in G1 cells, even though cell size had increased enormously (Fig. 3.1.2). The two cell samples were fixed, stained and measured together. This indicated that the mutant *cdG1-1* arrested without doubling its nuclear DNA at restrictive temperature.

Unlike mutant *cdG1-1*, three other mutants (*cdM-1*, *cdM-2* and *met-1*) revealed doubled nuclear DNA levels when arrested (Fig.3.1.3 and Fig.3.1.4). Again the samples from permissive and restrictive conditions for each mutant were fixed, stained and measured together. The nuclear DNA levels in G1 phase of synchronised cells of the *cdM-1*, *cdM-2* and *met-1* ranged respectively between 3-7, 3-13 and 5-9 units, and peaked respectively at 5, 6 and 7 relative units. In the arrested cells of the three mutants DNA contents of 6-14, 7-22 and 6-22 with peak values of 9, 10 and 16 units respectively, indicated that these mutants were blocked in G2 phase.

Another mutant, *cdCK-1,* was found to arrest as multinucleate cells at the restrictive temperature, and the number of nuclei in the arrested cells revealed a variety of 2, 4, 8 or even 16. Assaying the nuclear DNA amount in 50 single nuclei of the arrested cells demonstrated that the nuclear DNA level in each single nucleus was similar

to that of normal newly formed uninucleate daughters in G1 phase (Fig. 3.1.5). The result suggested that the mutant *cdCK-1* allows nuclear division and therefore return to G1 phase but prevents normal cytokinesis.

3.1.3.4. Study of the cell structure of the mutants

Analysis of the nuclear DNA level in arrested cells allowed a sorting of the five mutants into three classes of arrest phenotype: 1C, 2C and 1C-multinucleate. Further study of cell structure changes in the arrested cells was helpful for distinguishing their capacity for progress through the cytoskeletal changes of the normal cell cycle. In this work, study of cell structure was based on use of synchronous cultures especially in the case of the three mutants that were arrested with a 2C amount of nuclear DNA. For each mutant, synchronised early G1 daughter cells were transferred from permissive temperature to restrictive temperature at 0 h of the cell cycle. The cells were left at restrictive temperature to grow until they became fully arrested, normally after 20 h. Arrested cells were then labelled with antibody for immunofluorescence microscopy. Observations were focused on subcellular structures with important functions in cell division that could be detected by available antibodies such as anti-acetylated tubulin.

As a control, normal cell cycle progress in wild type *Chlamydomonas* (strain *CC-125*$^{+)}$ was also monitored by the same antibodies that were used for analysis of cdc mutants. The flagella and flagellar roots of *Chlamydomonas* cells contain stable microtubules that are acetylated posttranslationally on the lysine-ε-amino of the α-tubulin subunit which can be labelled by anti acetylated-tubulin antibody (Piperno and Fuller, 1985; Greer, 1985; Piperno et al., 1987; Wilson and Forer, 1989). In wild type *Chlamydomonas* cells, the anti-acetylated tubulin antibody stained not only the flagella and flagellar roots but also the basal bodies (L'Hernault and Rosenbaum, 1985; LeDizet and Piperno, 1986). Flagella regress before nuclear division (Gaffal, 1988), but a single flagellar root attached to each basal body remains to form, later in pre-prophase, the "metaphase band" which is at about 90° to the long axis of the spindle (Doonan and Grief, 1987; Gaffal and el-Gammal, 1990), persists through mitosis and may play a role

in directing the orientation of the cleavage furrow (Doonan and Grief, 1987; (Fig. 3.1.6)). During the cell cycle, the cortical microtubules were studied by indirect immunofluorescence microscopy using anti β-tubulin antibody as a probe. It was observed that early interphase cells contained a pair of flagella and 8-10 cortical microtubules that were focused in the region of the basal bodies and flagella roots. These microtubules increased in number as cells grew in size through G1 phase until the cells regressed their flagella after commitment to division. The cortical microtubules were depolymerised before formation of the mitotic spindle and were repolymerised after nuclear division (see Doonan and Grief, 1987 and Fig. 3.1.7). In parallel changes within the nucleus, chromatin became condensed to form chromosomes during early mitosis and moved to the metaphase plate when cells reached mitotic metaphase. Simultaneously, some mitosis specific proteins became phosphorylated and accumulated around the nuclear envelopes where they were detected by an MPM-2 antibody that had been raised against mitotic nuclei of the human HeLa cell line. This antibody has been used to detect mitosis-specific phosphoproteins around the nuclei of a wide range of eukaryotic cells including *Chlamydomonas* (Vandre et al., 1986; Harper et al, 1990a). When wild type cells were stained with anti MPM-2 antibody during G1 phase, there was no positive staining around the nuclei. When cells reached early prophase MPM-2 reacting proteins became detectable around the nucleus, seen as a spindle shaped outline as the cell entered metaphase and anaphase, which disappeared after mitosis (Fig. 3.1.8).

Another structure that is crucially involved in the cell cycle is the flagellar basal body apparatus or centrosome that is present in motile eukaryotic algae, and animal cells from protozoa to mammals (Salisbury et al., 1983; 1984; 1987). In *Chlamydomonas* centrin protein constitutes one of the components of the distal fibre which links the two adjacent components of the basal body pair to each other (McFadden et al., 1987; Salisbury et al., 1988; Bazinet et al., 1990; Salisbury, 1992) and also links the flagellar apparatus to the nucleus through a pair of descending fibres or connectors (Fig. 2.1.2). During interphase there is intense staining by anti-centrin of the connectors between the nucleus and basal bodies and more diffuse staining of the fimbrae that extend from the connectors around the nucleus (Wright et al., 1985). The centrin-based system becomes

contracted on the anterior region of the nucleus in cells approaching S phase as the flagella regress and nuclei move towards the flagellarapparatus. The centrin cytoskeleton then duplicates into two foci as the basal bodies duplicate and begin to separate towards the forming spindle poles during prophase (Fig. 3.1.9).

Mutant *cdG1-1* arrested as big spherical cells with 1C nuclear DNA content at restrictive temperature. When stained with PI, a single nucleus was observed in each arrested cell and the size of the single nucleus looked similar to that of the small newly-divided G1 cells that were grown at the permissive temperature (Fig. 3.1.10). Nomarski microscopy showed that the flagella of the arrested *cdG1-1* cells had regressed, while flagella of the newly divided cells formed at 21°C could be clearly observed (Fig. 3.1.11). Anti centrin antibody staining of the arrested cells revealed that the centrin cytoskeleton and basal bodies had not divided (Fig. 3.1.12). Cortical microtubules in the arrested cells, stained with anti β-tubulin antibody, had increased in number compared with that of the newly divided cells, but the cortical microtubules in the arrested cells remained in an interphase array except that they looked a little twisted and disorganised because of the block (Fig 3.1.13). No duplication of basal bodies or progress to spindle formation was detected in any of the arrested cells at the restrictive temperature (33°C). The interphase cell structure, together with the 1C nuclear DNA content, suggests that this mutant arrests in G1 phase of the cell cycle.

Mutants *cdM-1* and *cdM-2* had morphologically similar arrest phenotypes when incubated at restrictive temperature, although *cdM-1* cells were bigger, having a mean cell size of 900-1000 fl, whereas *cdM-2* arrested with a mean cell size of about 400 fl which is nonetheless five times the usual size of a daughter cell and not indicative of a major growth defect (Fig. 3.1.1 and Fig. 3.1.14). Arrested cells of the two G2 mutants did withdraw their flagella (Fig. 3.1.14). The cortical microtubules in the arrested cells of the two mutants appeared to have increased in number and became a little bit disorganised, as also occurred in the G1-arresting mutant cells. Although cells of the two G2-arresting mutants were able to proceed normally up to the G2 phase of the cell cycle by doubling nuclear DNA there was no indication of any progress towards spindle formation in any arrested cells (Fig. 3.1.15, Fig. 3.1.16). Confirmation of the

observation by Nomarski microscopy that flagella had been withdrawn was provided by immunofluorescence microscopy using anti-acetylated tubulin antibody, which showed that arrested cells did succeed in withdrawing their flagella before they became arrested at the restrictive temperature. However, neither duplication nor separation of the basal bodies was observed in the arrested cells (Fig 3.1.17). Anti centrin antibody staining of the *cdM-2* mutant cells also revealed arrest with an undivided centrin cytoskeleton (Fig. 3.1.17). In conformity with cytoskeletal evidence that the G2/M boundary had not been traversed, no MPM-2 staining was detected in arrested cells of these two mutants. Therefore, although the nuclear states had reached a 2C level of DNA, the cell structure in these two mutants indicated that they had not entered mitosis.

The mutant *met-1* showed a distinctive arrest phenotype at the restrictive temperature. Doubled nuclear DNA content in the arrested cells of this mutant became condensed to form chromosomes aligning at the metaphase plate. The cortical microtubules disassembled and reorientated to form a full mitotic metaphase spindle, but no further progress could occur to allow cells to enter anaphase (Fig 3.1.18.). Interestingly, many of the arrested *met-1* cells had attempted initiation of their cytoplasmic cleavage furrows (Fig. 3.1.18b), whereas cells cultured at the permissive temperature of 21°C were able to pass through the metaphase/anaphase transition and completed the cell cycle normally (Fig. 3.1.19). When the arrested *met-1* cells were stained with anti-acetylated antibody, no flagella were observed in any arrested cells and basal bodies in most arrested cells were duplicated and had separated to the spindle poles, which were connected to the accurately placed "metaphase bands" (Fig 3.1.20). Surprisingly, spindle microtubules in the regions close to the poles in the arrested cells were found to be acetylated (Fig 3.1.20). This was not clearly observed in the wild type cells or in mutant cells that were incubated at permissive temperature, in both of which the spindle was present only briefly and the anti acetylated tubulin antibody only stained the "metaphase band" (Fig. 3.1.6. and Fig 3.1.20).

Mitotic arrest was further confirmed by MPM-2 antibody staining. When cells were grown at permissive temperature, only the cells that were about to initiate or had initiated mitosis showed positive MPM-2 staining, as previously observed in wild type

Chlamydomonas by Harper et al. (1990). When cells were grown at the restrictive temperature, however, almost all cells blocked with bright staining of mitotic phosphoprotein(s) around their nuclear envelope (Fig 3.1.21).

The *cdCK-1* mutant could go through the cell cycle normally at 21°C (Fig.3.1.22), but when incubated at 33°C, the mutant arrested in multinucleate form. Immunofluorescence microscopy using anti acetylated-tubulin antibody revealed that basal bodies in the multinucleate arrested cells were duplicated and were well separated together with the daughter nuclei, but the cells were not able to form normal phycoplasts of tightly aggregated microtubules (Fig. 3.1.23). Staining with anti β-tubulin antibody revealed that interphase cortical microtubules had disappeared from arrested cells . This presumably correlates with their depolymerisation prior to successful formation of a mitotic spindle and completion of nuclear division. Thus a number of major cytoskeletal changes were successfully negotiated, but the next scheduled cytoskeletal event, formation of a phycoplast, was not attained (Fig. 3.1.24). Although microtubules were deployed between daughter nuclei, approximately in the location of a phycoplast, they were not tightly grouped in the close transverse arrangement that is characteristic of phycoplasts (Fig. 3.1.24).

3.1.3. Discussion

Five temperature sensitive cell division cycle mutants have been characterised in this work. Each of these mutants has identified a single gene function that can cause arrest in a particular stage of the cell cycle. Backcrossing has established that in each case a single gene mutation is responsible for the phenotype and repeated backcrossing has obtained each mutation in a wild type background. Investigation of the continuation of growth and division under restrictive conditions has indicated that the primary effect of the mutations is to arrest division, not growth, since cells at restrictive temperature can grow prior to and after arrest. At the permissive temperature cells of each mutant underwent normal division indistinguishable from wild type. On shifting to the restrictive

temperature in early G1 phase, these five temperature-sensitive mutants became arrested respectively in G1 phase, G2 phase, mitotic metaphase and cytokinesis of the cell cycle. The terminal phenotype of each mutant has been established in terms of their cell structure and nuclear DNA levels in the arrested cells as summarised in Table 3.2.

Table 3.2. Terminal phenotypes of the five cell division cycle mutants at 33°C

Mutant	*cdG-1*	*cdM-1*	*cdM-2*	*met-1*	*cdCK-1*
Flagella regression	yes	yes	yes	yes	yes
Number of nuclei per cell	1	1	1	1	>2
Nuclear DNA level per nucleus	1C	2C	2C	2C	1C
Cortical microtubule depolymerisation	no	no	no	yes	yes
Centrin connector contraction	no	no	no	yes	yes
Basal body duplication	no	no	no	yes	yes
Chromosome condensation	no	no	no	yes	yes
Metaphase band formation	-	-	-	yes	yes
Spindle formation	-	-	-	yes	yes
Acetylation of spindle microtubules	-	-	-	yes	?
Spindle elongation	-	-	-	no	yes
Daughter nuclei formation	-	-	-	-	yes
Cytokinesis completion	-	-	-	-	no
Phases in which cells arrest	G1	G2	G2	mitosis	cytokinesis

Mutant *cdG1-1* arrested with undoubled nuclear DNA and non disassembled interphase cortical microtubules, even though the cells of this mutant had withdrawn their flagella when blocked at 33°C. This mutation resulted in failure to replicate nuclear DNA and it has been observed in all eukaryotes that have been investigated that a strong checkpoint prevents initiation of mitosis in cells that have not completed nuclear DNA replication (Murray,1992). Because of the block, spindle formation was not observed in this mutant. Neither did initiation of cytokinesis occur in arrested cells. This G1-

arresting mutant identifies a gene function that is necessary for DNA replication. The withdrawal of flagella indicates that commitment to division has been attained and this is examined further in subsection 3.2.

Whereas, mutants *cdM-1* and *cdM-2* were blocked in entry into M phase of the cell cycle. The arrested cells could grow normally, pass through the commitment point and had succeeded in withdrawing their flagella (Fig. 3.1.10). Terminal phenotypes of the two mutants are very similar, but they arrested with different cell size. The *cdM-1* cells arrested with a mean cell size of about 900 to 1000 fl (Fig. 3.1.1), which is bigger than that of all other mutants, whereas *cdM-2* arrested at 400 fl mean cell size which is a common size for mother cells of wild type but the smallest among the five mutants (Fig. 3.1.1). At restrictive temperature, both *cdM-1* and *cdM-2* cells were able to double their nuclear DNA, but neither were able to disassemble their interphase cortical microtubules, nor to form mitotic spindles. These two mutants have been isolated independently, therefore there is little possibility that they both reside in a gene that is responsible for disassembly of the cortical microtubules. There is a small chance that the mutations inactivate two different proteins that are both required for cytoskeletal change. However it seems more likely that the proteins altered by mutation are among the large number that are presumably required for the major transition between G2 phase and M phase. In accord with this both mutants were found to allow only partial activation of the $p34^{cdc2}$-like kinase that initiates many mitotic events, as will be described in section 3.3. This, on the other hand, for the first time specifically suggests that depolymerisation of the cortical microtubules in *Chlamydomonas* can be identified as a late G2 phase event that is part of the initiation of mitosis.

The mutant *met-1* is a metaphase-arresting mutant by the following criteria: (1) the arrested cells succeeded in regression of flagella and depolymerisation of the interphase cortical microtubules, which indicates that the cells had initiated mitosis; (2) a spindle formed; (3) abundant phosphorylated proteins recognised by MPM-2 antibody have been detected around the nuclear envelope of the *met-1* arrested cells. These phosphoproteins only become abundant around the nucleus during mitosis, in wild type or mutant cells cultured at permissive temperature; (4) chromatin in the arrested cells is

condensed to form chromosomes that are aligned at the metaphase plate of a fully formed mitotic spindle in a conformation indistinguishable from mitotic metaphase; and (5) it has been noted that the mitotic spindle microtubules in the *met-1* arrested cells were partially acetylated (Fig. 3.1.20).

Acetylation of the mitotic spindle in arrested *met-1* cells is unlikely to be the primary cause of the metaphase arrest, rather it may be a consequence, since two tubulin mutants in *Chlamydomonas* (*col*^{R}*14* and *col*^{R}*15*) that contain acetylated cortical and mitotic spindle microtubules have wild type characteristics and do not express the lethal phenotype of the cell cycle arrests (Schibler and Huang, 1991). This finding indicates that spindle acetylation need not block nuclear division. Moreover, acetylation of spindles has been observed to occur to varied extents in different cell types, from none in *Drosophila* embryos (Wolf et al., 1988) to limited acetylation of only the polar region in mouse oocytes and *Physarum* (Schatten et al., 1988; Sasse et al., 1987) to general staining of spindle microtubules in some cultured mammalian cells and in trypanosome (Piperno et al., 1987; Cambry-Deakin and Burgoyne, 1987; Sasse and Gull, 1988). It has been suggested that during metaphase tubulin subunits are added at the kinetochore and become acetylated at some time afterwards, therefore the tapered area can be considered a microtubule marker for anaphase chromosome motion (Wilson and Forer, 1989). The spindle acetylation seen in the *met-1* arrested cells can be most simply accounted for as resulting from the establishment of metaphase followed by the slow acetylation of proteins in the persisting spindle. Thus, the *met-1* mutant identifies a gene function that is necessary for the metaphase/anaphase transition.

The mutant *cdCK-1* is arrested in cytokinesis. Nuclear division can occur normally in *cdCK-1* cells at restrictive temperature but cytokinesis is blocked, which results in a multinucleate cdc phenotype. The microtubule-based cortical cytoskeleton depolymerised normally at mitosis at the restrictive temperature and after nuclear division the microtubules were able to align approximately between the daughter nuclei as expected for a future phycoplast (Fig.3.1.24), but the tight focusing of the microtubules that is normally involved in establishing a phycoplast was unable to occur.

This mutant identifies a gene function that is necessary for completion of cytokinesis and is probably involved in phycoplast establishment and cleavage furrow formation.

Isolation and characterisation of these mutants have proved that *Chlamydomonas* is a good experimental subject for isolation of a whole range of temperature sensitive cell division cycle mutants that are defective in G1, G2, M and cytokinesis phases. The analysis of the terminal phenotypes of the mutants here allowed only a primary classification of the mutants into major phases of the cell cycle. Further analysis of each mutation (except the more simply interpretable *cdCK-1* mutant) are described in the following sections.

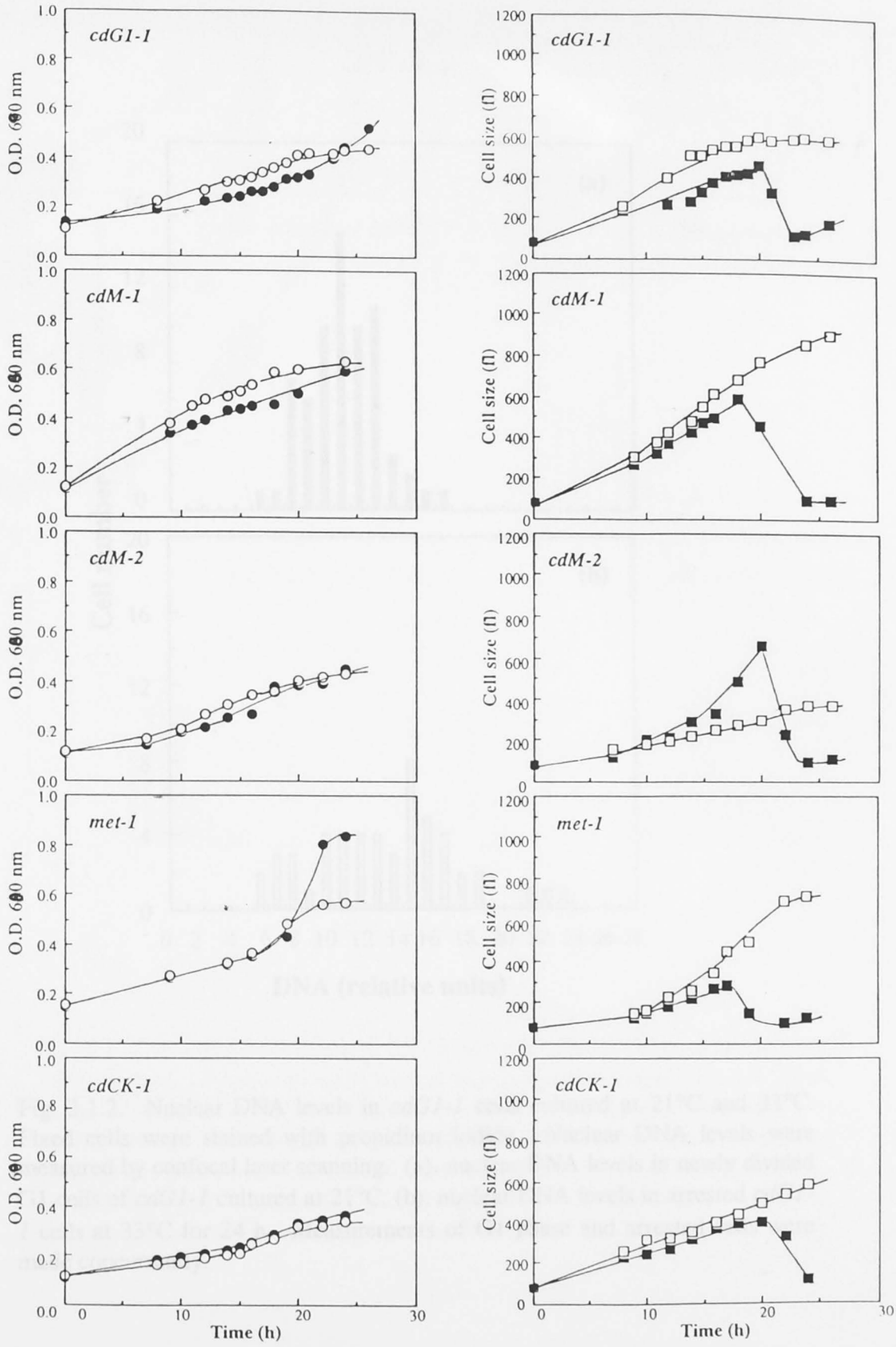


Fig. 3.1.1. Culture turbidity (circles) and mean cell size (squares) in synchronous cultures of the five mutants grown at 21°C (closed circles and squares) and 33°C (open circles and squares). Sampling was started at the beginning of growth in newly formed daughter cells. Turbidity of each sample was determined by measuring the O.D. 680nm and mean cell size was measured by Coulter counter. (●), cell turbidity of the cultures at 21°C; (○), cell turbidity of the cultures at 33°C; (■), mean cell size of the cultures at 21°C; (□), mean cell size of the cultures at 33°C.

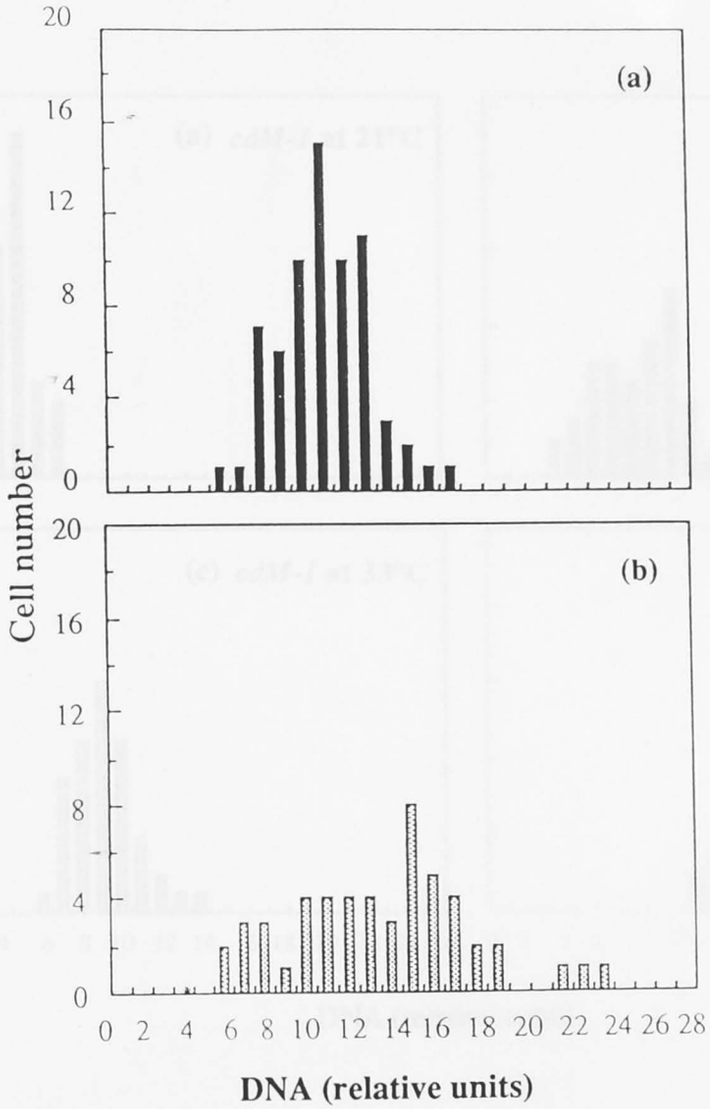


Fig. 3.1.2. Nuclear DNA levels in *cdG1-1* cells cultured at 21°C and 33°C. Fixed cells were stained with propidium iodide. Nuclear DNA levels were measured by confocal laser scanning. (a), nuclear DNA levels in newly divided G1 cells of *cdG1-1* cultured at 21°C; (b), nuclear DNA levels in arrested *cdG1-1* cells at 33°C for 24 h. Measurements of G1 phase and arrested cells were made concurrently.

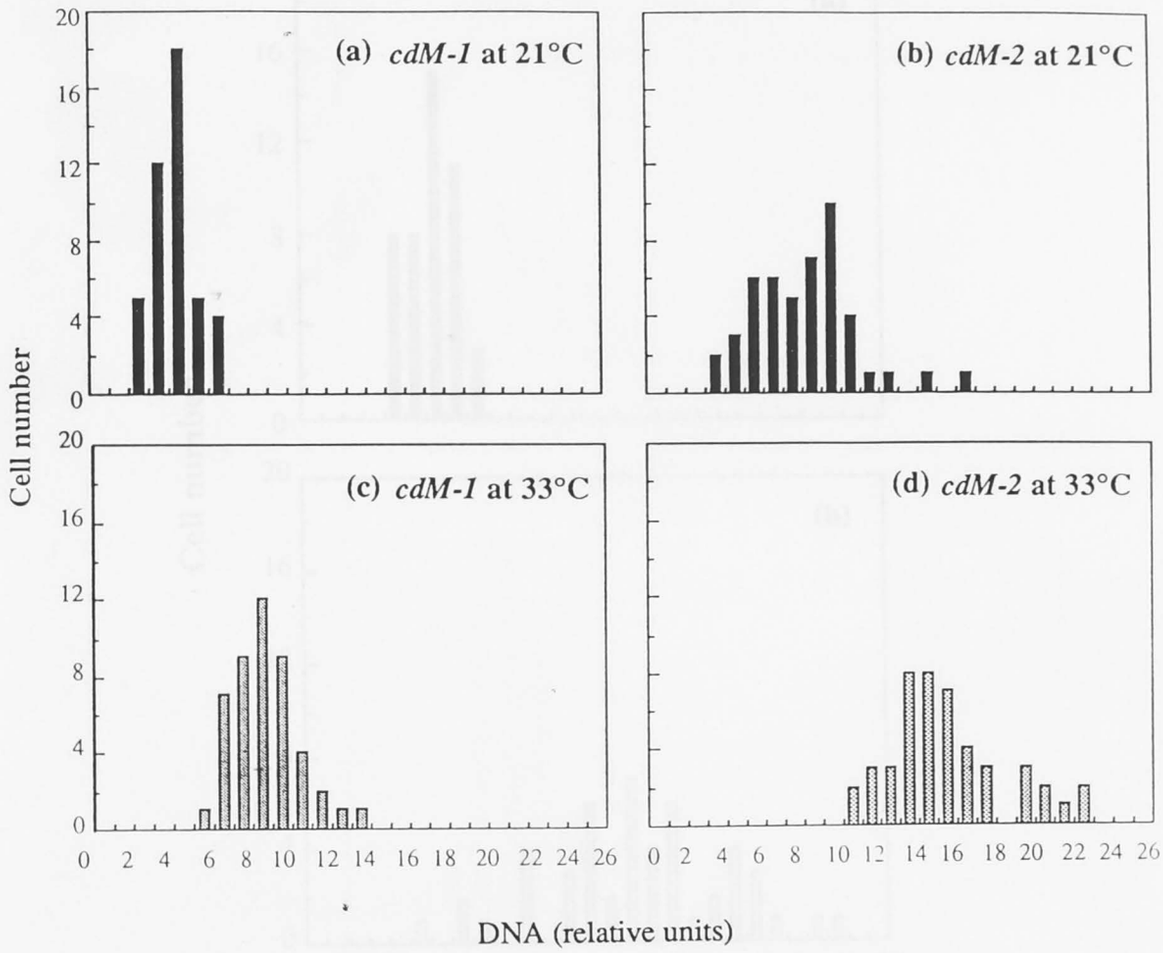


Fig. 3.1.3. Nuclear DNA levels in *cdM-1* and *cdM-2* cells cultured at 21°C and 33°C for 24 h. Fixed cells were stained with propidium iodide. Nuclear DNA levels were measured by confocal laser scanning (see General Method 2.2.9) (a)(b), nuclear DNA levels in newly divided G1 cells of the two mutants cultured at 21°C; (c)(d), nuclear DNA levels in arrested cells of the two mutants at 33°C for 24 h. Measurements of G1 phase and arrested cells of individual mutants were made concurrently.

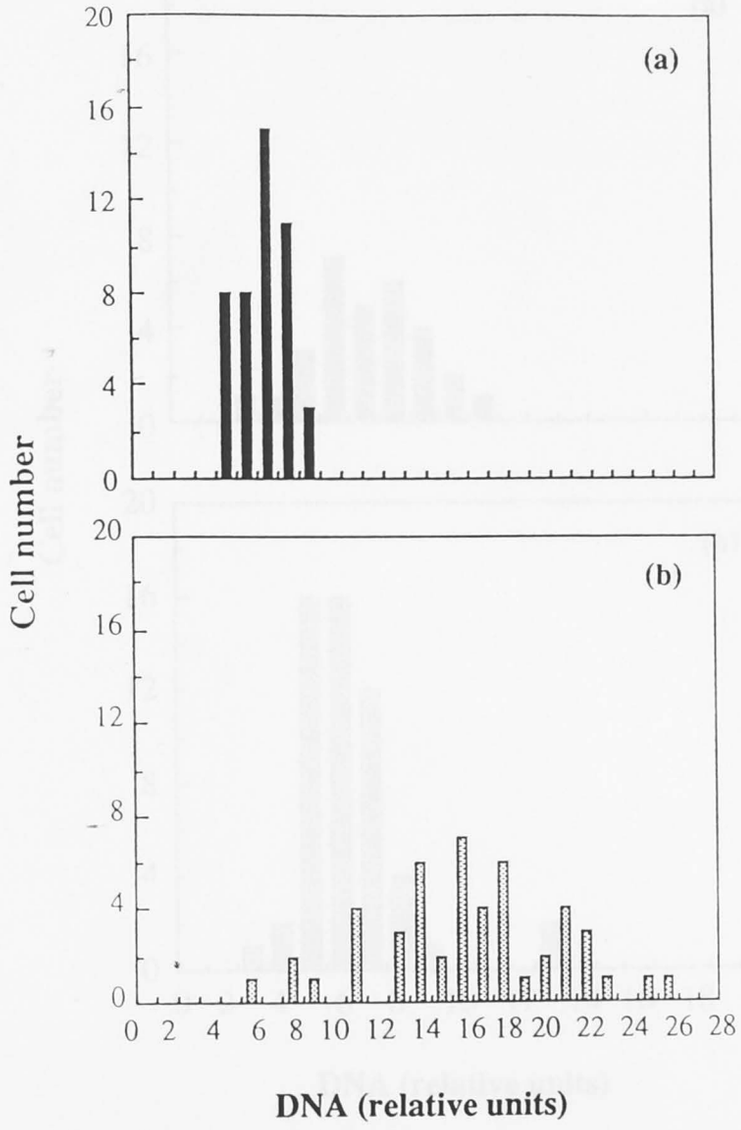


Fig.3.1.4. Nuclear DNA levels in *met-1* cells cultured at 21°C and 33°C for 24 h. Fixed cells were stained with propidium iodide. Nuclear DNA levels were measured by confocal laser scanning (see General Method 2.2.9). (a), nuclear DNA level in newly divided early G1 cells of *met-1* cultured at 21°C; (b), nuclear DNA level in arrested *met-1* cells that were cultured at 33°C for 24 h. Measurements of G1 phase and arrested cells were made concurrently.

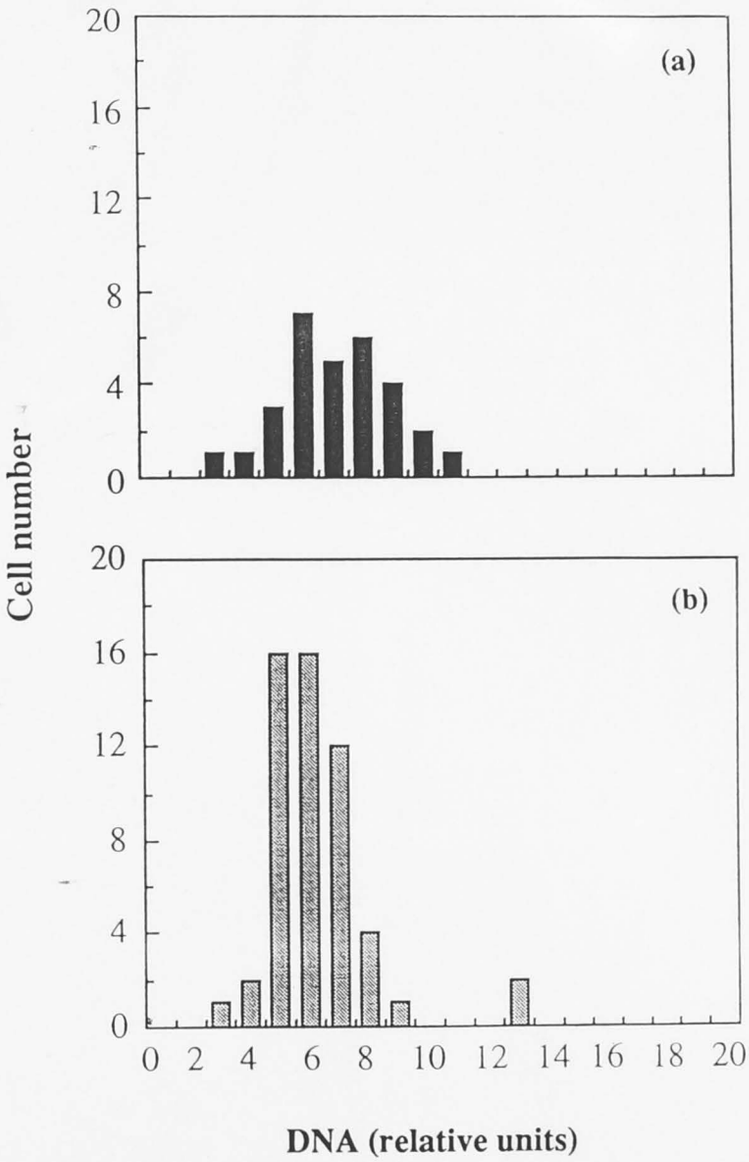


Fig. 3.1.5. Nuclear DNA levels in *cdCK-1* cells cultured at 21°C and 33°C for 24 h. Fixed cells were stained with propidium iodide. Nuclear DNA levels were measured by confocal laser scanning (see General Method 2.2.9). (a), nuclear DNA level in newly divided G1 cells of *cdCK-1* cultured at 21°C; (b), nuclear DNA level in arrested cells of *cdCK-1* cultured at 33°C. Measurements of G1 phase and arrested cells were made concurrently.

Fig. 3.1.6. Anti acetylated-tubulin antibody staining of the wild type *Chlamydomonas* cells (*CC-125*$^{+}$), showing dynamics of the acetylated microtubules during a normal cell cycle.

(a), a newly divided cell stained with anti acetylated-tubulin antibody, which detects the flagella roots and the proximal portion of the cortical microtubules of the cell.

(b), DAPI staining of the cell in (a), showing the nucleus (arrow) in the anterior end of the cell.

(c), two cells in prophase of the third mitosis, which will form 8 daughters. The mother cell is seen in prophase and although four DAPI stained nuclei can be seen (d) the associated cytoskeleton is in focus for only two of the nuclei. Anti acetylated-tubulin antibody staining revealed that the cells had withdrawn their flagella and each cell had formed a "metaphase band (MB)" (between the tips of the large arrow heads). Note that the MB is outside the nucleus and is formed by residual root microtubules that emanate from each pole (small arrow heads). Where the two residual roots are in antiparallel contact close to their distal ends (between the pairs of large arrow heads) their alignment is approximately at 90° to what will be the long axis of the nucleus and spindle. The angle made with the spindle axis is variable, as seen by comparing (c) and (e).

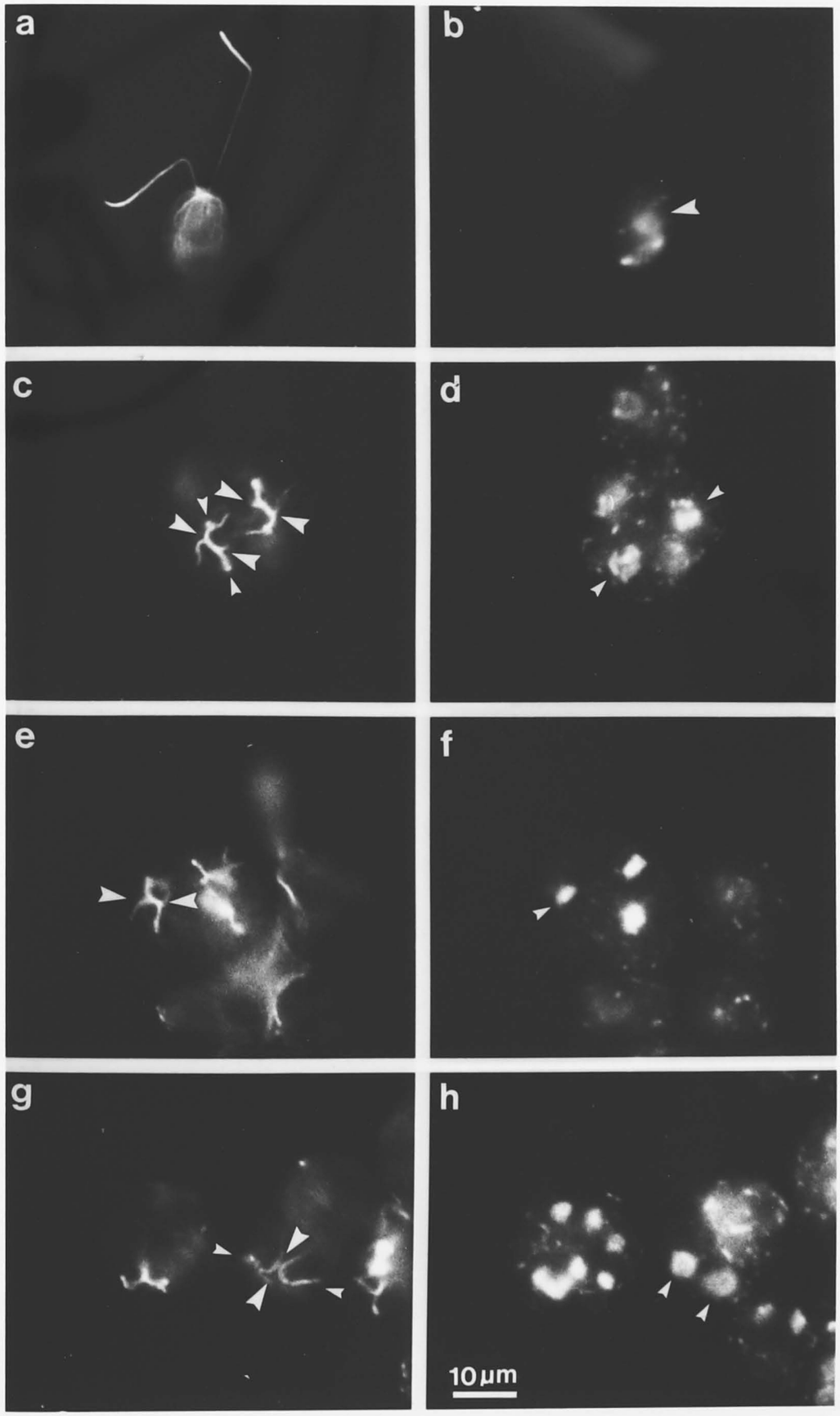
(d), DAPI staining of the cell in (c), showing the prophase nuclei of the two cells with condensed chromosomes.

(e), anti acetylated tubulin antibody stained MB (between large arrow heads) present in cells at metaphase.

(f), DAPI staining of the cells in (e), the DAPI stained chromosomes were aligned at the metaphase plates.

(g), cell in telophase stained with anti acetylated tubulin antibody, showing that with elongation of the spindle the length of contact of the roots in the antiparallel MB region is reduced (between the large arrow heads).

(h), DAPI staining of the cell in (g), the chromosomes have moved towards the spindle poles and have started to form daughter nuclei (small arrow heads).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b
c
d
e
f
g
h
10 µm

Fig. 3.1.7. Anti β-tubulin antibody staining of wild type *Chlamydomonas* cells (*CC-125*$^{+}$) showing dynamics of the cortical microtubules during a normal cell cycle.

(a), four newly-divided cells; the antibody stained the flagella and cortical microtubules.

(b), DAPI staining of the cells in (a), showing the position of the nucleus in each cell (arrows).

(c), three cells which had grown at 21°C for 12 h; the anti β-tubulin antibody stained the flagella and abundant cortical microtubules. One of the cells (bottom left) was withdrawing its flagella in preparation for division.

(d), corresponding DAPI staining of the cells in (c), the nuclei (arrowed) in these three cells were still in interphase.

(e), cell in metaphase; the β-tubulin antibody stained the metaphase spindle and no cortical microtubules were detectable.

(f), DAPI staining of the cell in (e), showing that the chromosomes had aligned at the metaphase plate.

(g), a cell in anaphase with a anti β-tubulin stained anaphase spindle, the central region of the spindle is here not obscured by chromosomes as was the case at metaphase.

(h), DAPI staining of the cell in (g), showing the chromosomes that were moving towards the spindle poles.

(i), anti β-tubulin staining showing eight newly formed daughter cells before release from the mother cell wall.

(j), newly released daughter cells stained with anti β-tubulin antibody.

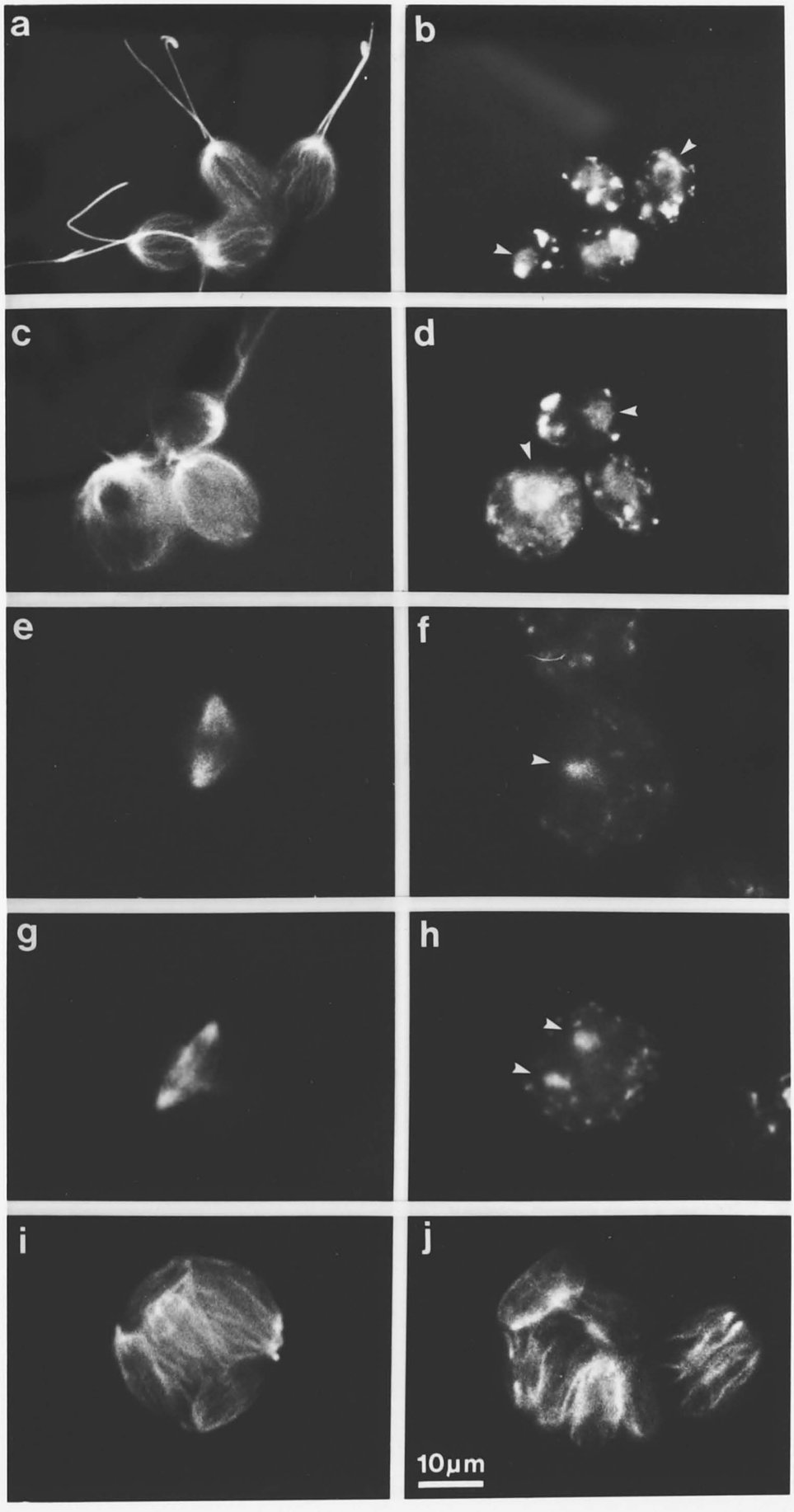
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Fig. 3.1.8. Anti MPM-2 antibody staining of the wild type cells (*CC-125*$^{+}$), showing the changing distribution of MPM-2 proteins during a normal cell cycle of *Chlamydomonas*.

(a), G1 phase cell stained with anti MPM-2 antibody; no positive staining was revealed in the cell.

(b), DAPI staining of the cell in (a), showing the position of the nucleus in the cell.

(c), cell in prophase with MPM-2 positive staining in the area of the nucleus.

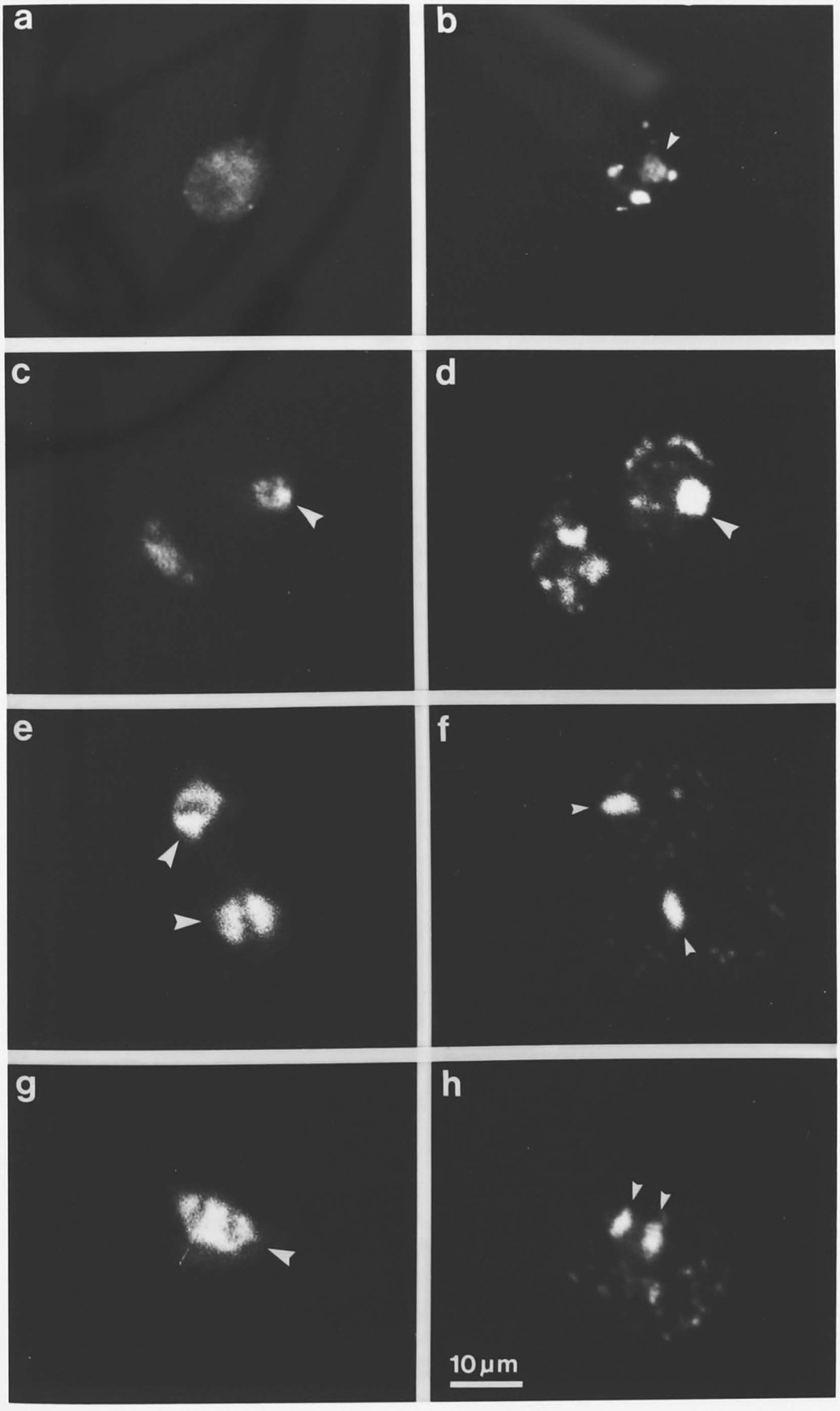
(d), DAPI staining of the cell in (c), showing the condensed chromatin.

(e), two cells in metaphase, the anti MPM-2 antibody stained proteins around the spindle. The plane of focus is on the lower surface of the nuclei and a dark band due to the metaphase chromosomes is seen centrally.

(f), DAPI staining of the cells in (e), showing the position of the metaphase chromosomes at the metaphase plate.

(g), anaphase cell stained with anti MPM-2 antibody; abundant MPM-2 proteins were detected by the antibody forming a halo around the elongated anaphase spindle. Two dark bands are seen due to the two sets of migrating chromosomes seen in (g) shading signal from the lower surface of the nucleus.

(h), DAPI staining of the cell in (g), showing chromosomes that were moving towards the spindle poles.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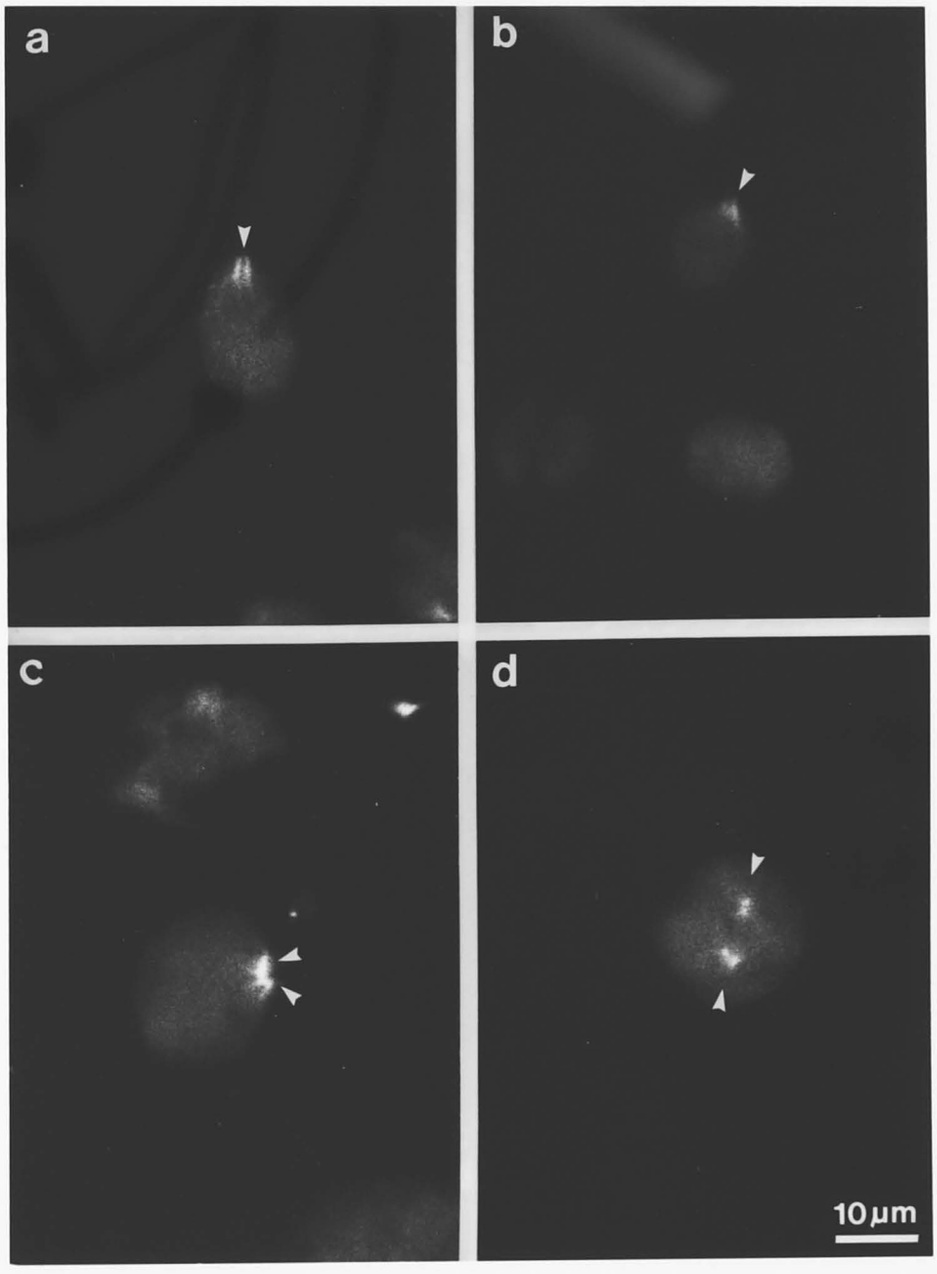


Fig. 3.1.9. Anti centrin staining of wild type *Chlamydomonas* cells (*CC-125*$^{+}$), showing the changes of centrin-containing cytoskeleton during cell cycle. (a), an early G1 phase cell with intense staining of the two fibers between the nucleus and the basal bodies (arrow) and more diffused staining of the fimbriae around the nucleus; (b), an early pre-prophase cell with contracted centrin-based fiber system forming a tight aggregate of material on the anterior of the nucleus. The nucleus has been moved, by contraction of the connector, closer to the flagellar apparatus from which the basal bodies will act as spindle poles; (c), the centrin cytoskeleton in the prophase cell divided into two foci (arrows); (d), the two centrin foci moved with the basal bodies to the opposite spindle poles at metaphase.

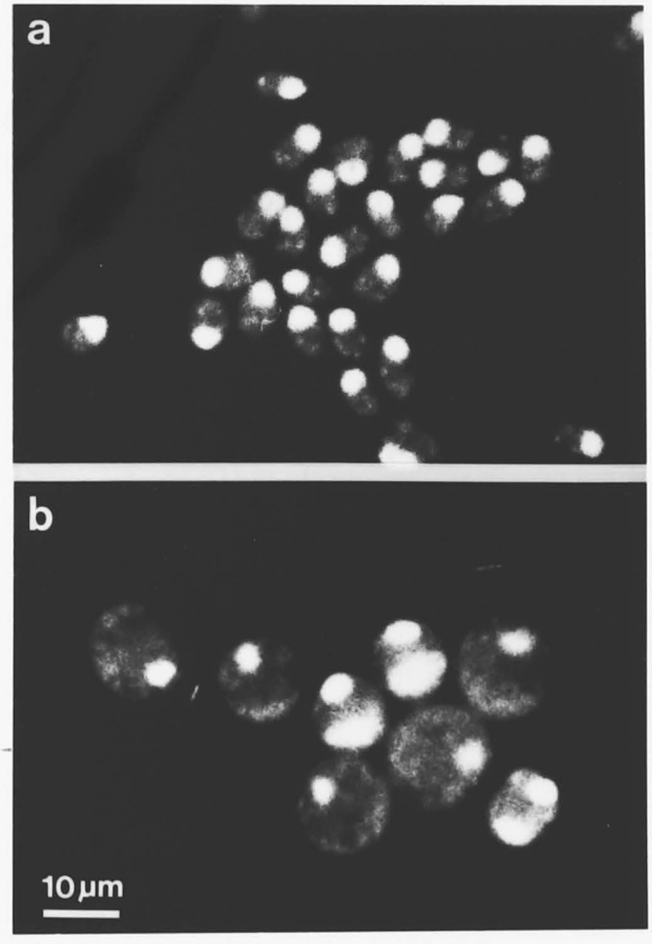


Fig. 3.1.10. Propidium iodide staining of the *cdG1-1* cells cultured at 21°C and 33°C for 24 h. (a), cells cultured at 21°C and sampled in early G1 phase, each cell contains a single nucleus; (b), cells that had been cultured at 33°C for a further 24 h were arrested and contained only a single nucleus although they had grown to five times as large (Fig. 3.1.1).

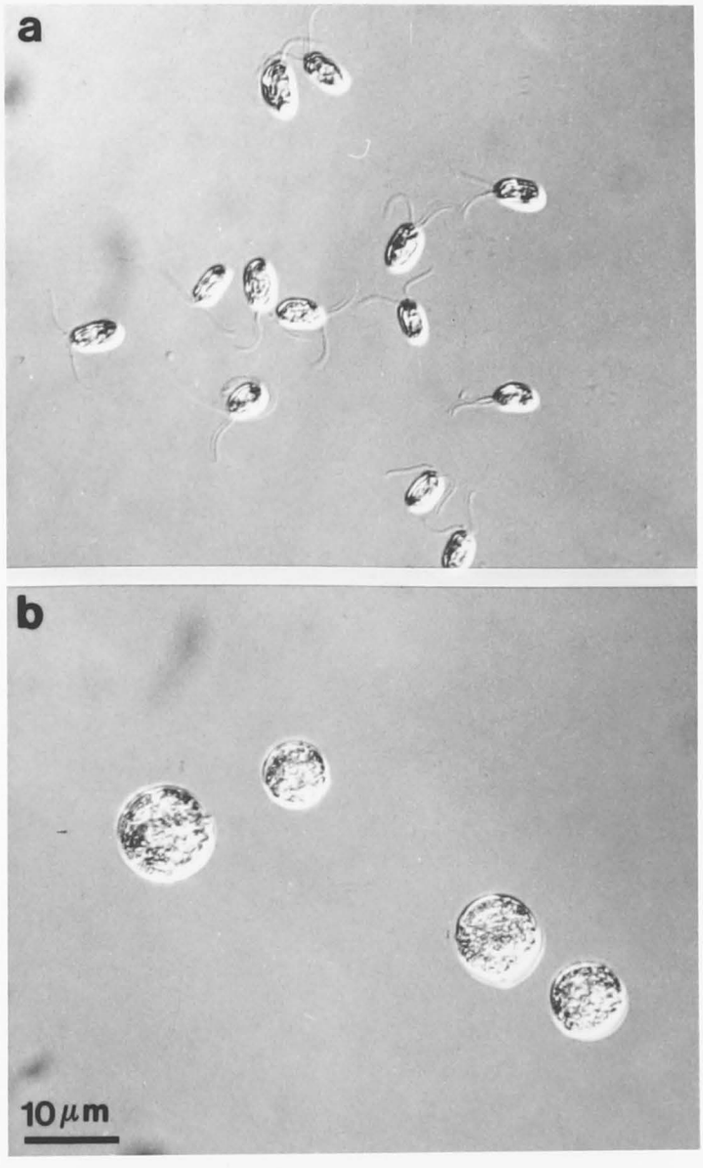


Fig. 3.1.11. The G1-arresting mutant (*cdG1-1*) cultured at 21°C and 33°C, showing regressed (absent) flagella in arrested cells. Synchronous cells were incubated in parallel at 21°C and 33°C from the beginning of the cell cycle at 0 h and were fixed with 1% formaldehyde at 24 h. The fixed cells were observed by Nomarski microscopy. (a), cells cultured at 21°C for 24 h; (b), cells cultured at 33°C for 24 h.

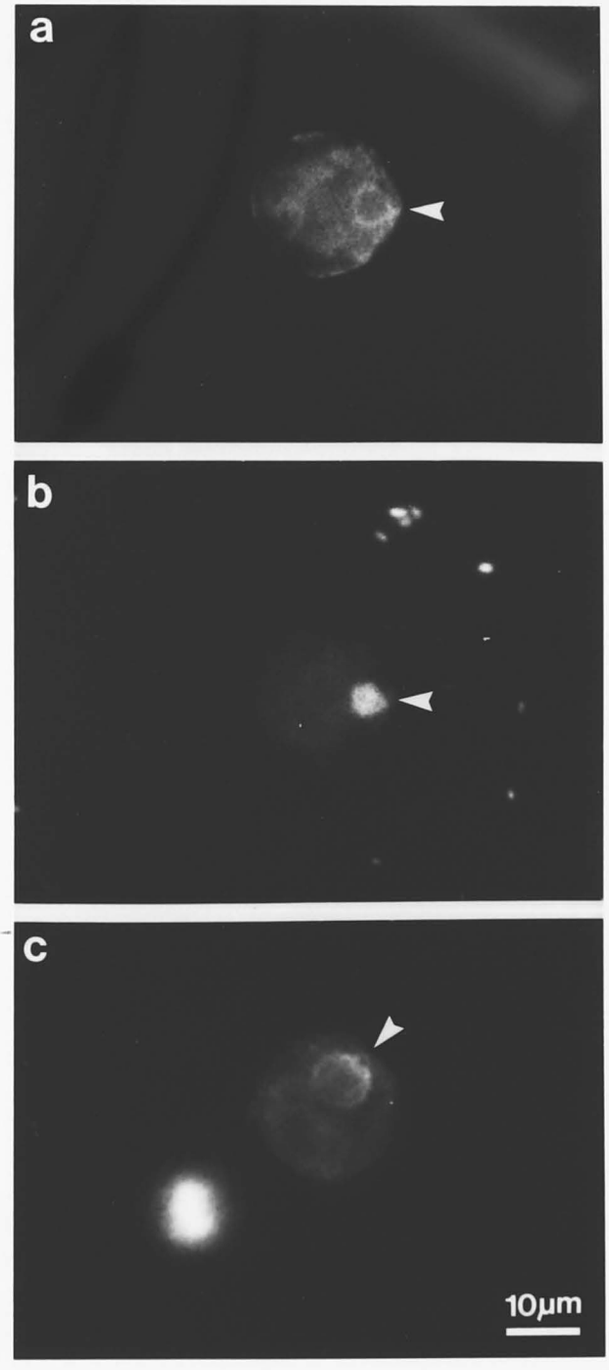


Fig. 3.1.12. Anti centrin staining of *cdG1-1* cells cultured at 33°C for 24 h. (a), cell stained with anti centrin antibody, detecting connectors between the nucleus and the basal body (arrow) and also the fimbria faintly outlining the nucleus, which revealed the interphase characteristics of the centrin cytoskeleton; (b), DAPI staining of the cell in (a), showing the position of the nucleus; (c), another arrested cell stained with anti centrin antibody showing the centrin-containing connectors in extended interphase configuration (arrow).

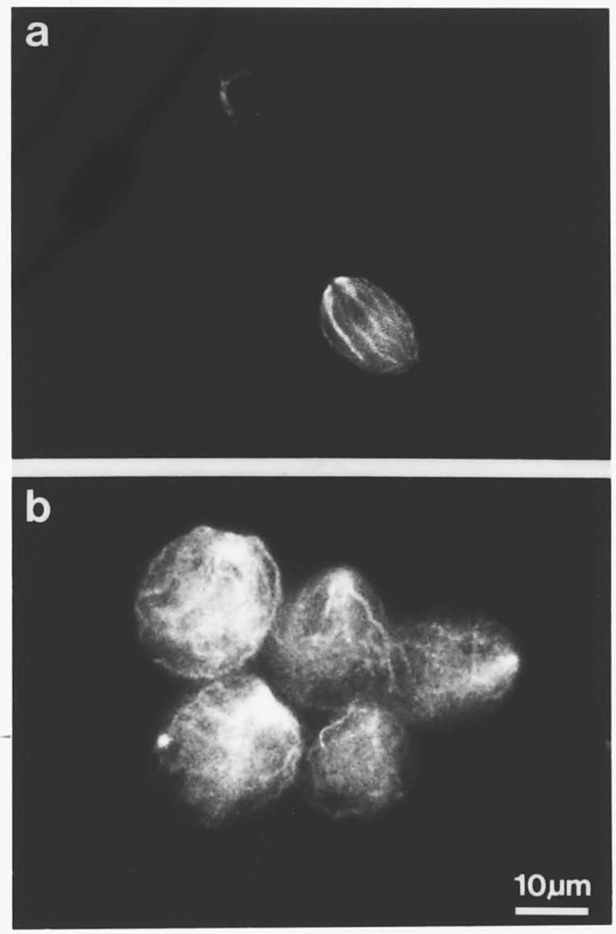


Fig. 3.1.13. Anti β-tubulin antibody staining of the *cdG1-1* cells cultured at 21°C and 33°C. (a), cell cultured at 21°C, the antibody stained the cortical microtubules and the flagella of the cell; (b), arrested *cdG1-1* cells that were cultured at 33°C for 24 h, showing the interphase cortical microtubules maintained in the arrested cells although these were 4-5 times larger than normal size (Fig. 3.1.1).

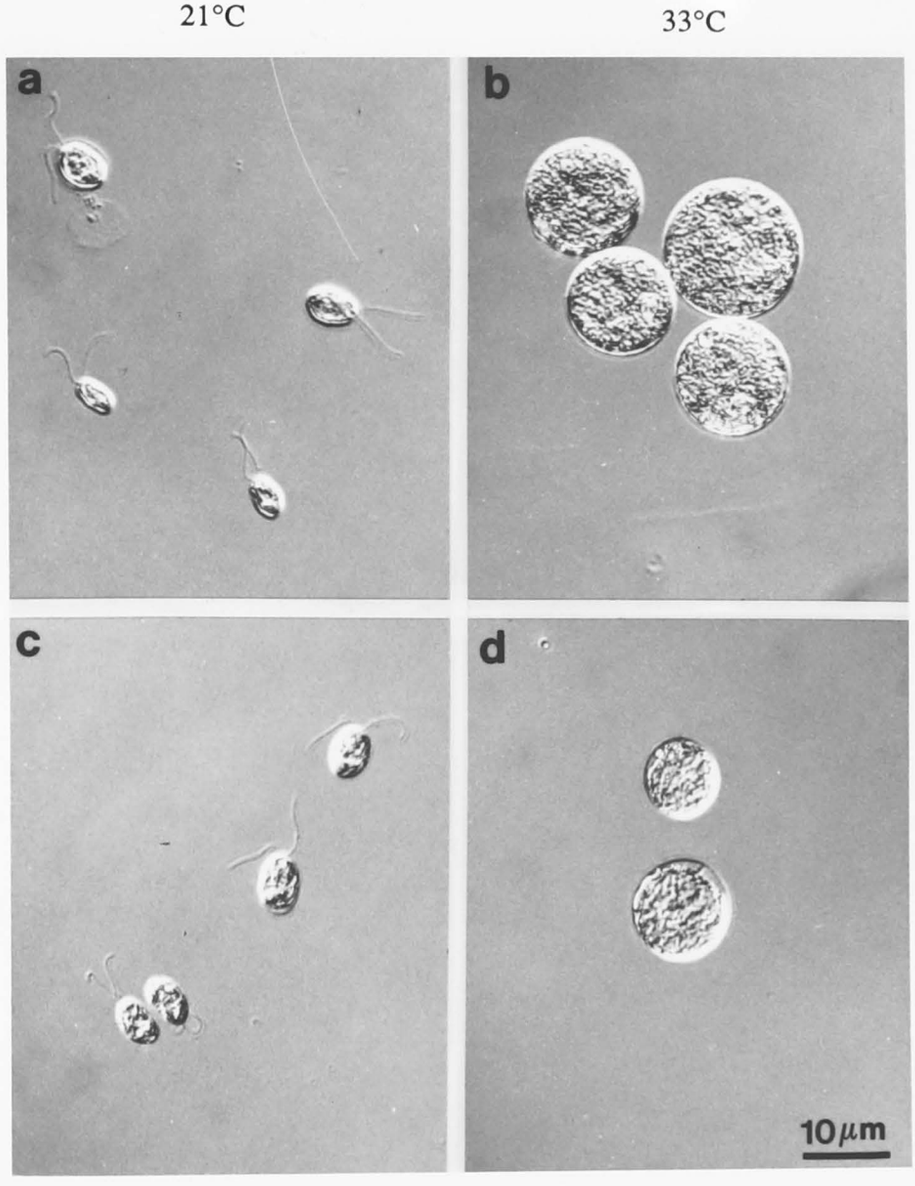


Fig.3.1.14. The G2-arresting mutants (*cdM-1* and *cdM-2*) cultured at 21°C and 33°C, showing the withdrawn flagella of the arrested cells. Synchronous cells of the two mutants were incubated in parallel at 21°C and 33°C from the beginning of the cell cycle at 0 h. The cells were fixed with 1% formaldehyde after 24 h and observed by Nomarski microscopy. (a), *cdM-1* cells cultured at 21°C; (b), *cdM-1* cells arrested at 33°C; (c), *cdM-2* cells cultured at 21°C; (d), *cdM-2* cells arrested at 33°C.

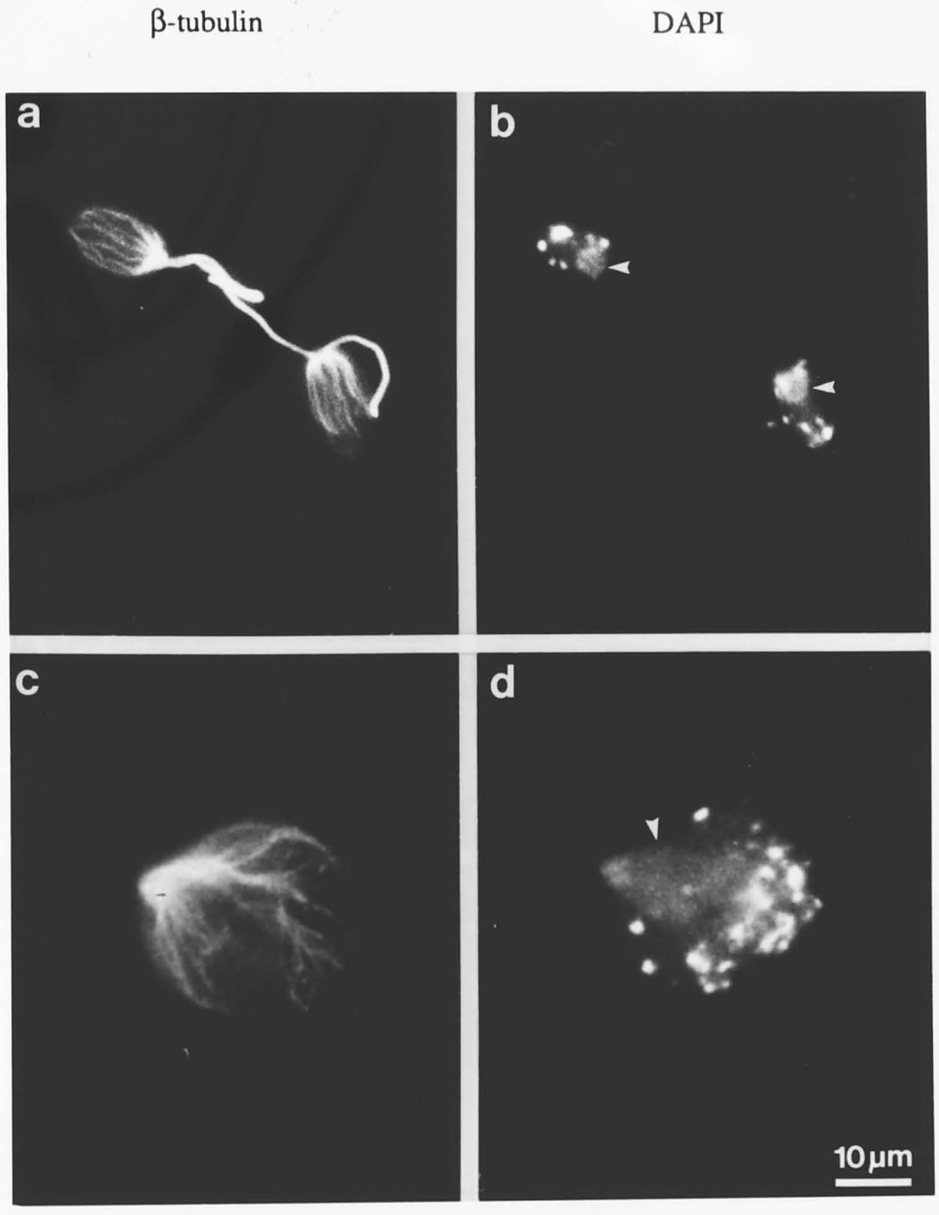


Fig. 3.1.15. Anti β-tubulin staining of the *cdM-1* cells cultured at 21°C (a, b) and 33°C (c, d). (a), anti β-tubulin staining of two newly-divided cells that had previously been cultured at 21°C for 24 h. These two cells were clearly ellipsoid. The antibody stained cortical microtubules and flagella; (b), DAPI staining of the cells in (a), showing the single nucleus; (c), anti β-tubulin staining of one of the arrested cells that had been cultured at 33°C for 24 h, showing the intact interphase cortical microtubules which had increased in number from the beginning of the cell cycle. The cells were nine times as big as normal daughter cells (Fig. 3.1.1) and were in G2 phase (Fig. 3.1.3). The flagella had regressed in the arrested cell; (d), DAPI staining of the cell shown in (c), showing the nucleus (arrow).

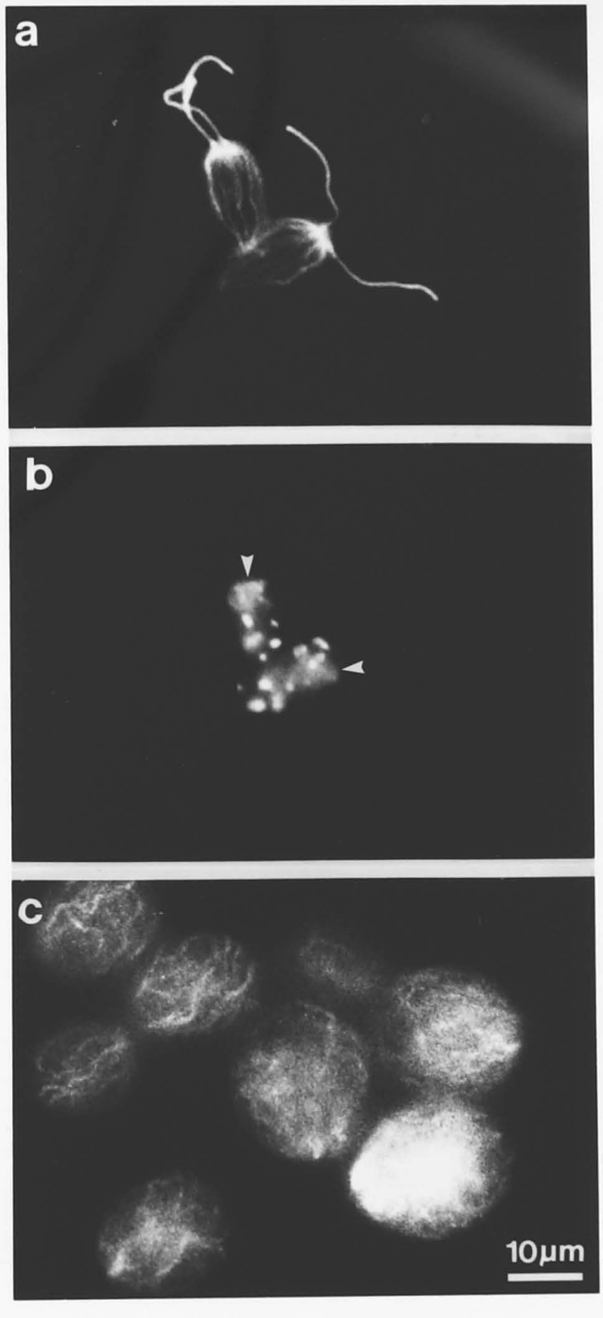


Fig. 3.1.16. Anti β-tubulin staining of *cdM-2* mutant cells cultured at 21°C and 33°C. (a), anti β-tubulin antibody staining of two newly-divided cells cultured at 21°C for 24 h. The cells were ovoid in shape, the antibody stained cortical microtubules and flagella; (b), DAPI staining of the cells in (a), showing the position of the nucleus in each cell; (c), anti β-tubulin antibody staining of arrested cells cultured at 33°C, showing the cortical microtubules which had increased in number as the cell enlarged four fold (Fig. 3.1.1) and persisted in these blocked cells that were arrested in G2 phase (Fig. 3.1.3).

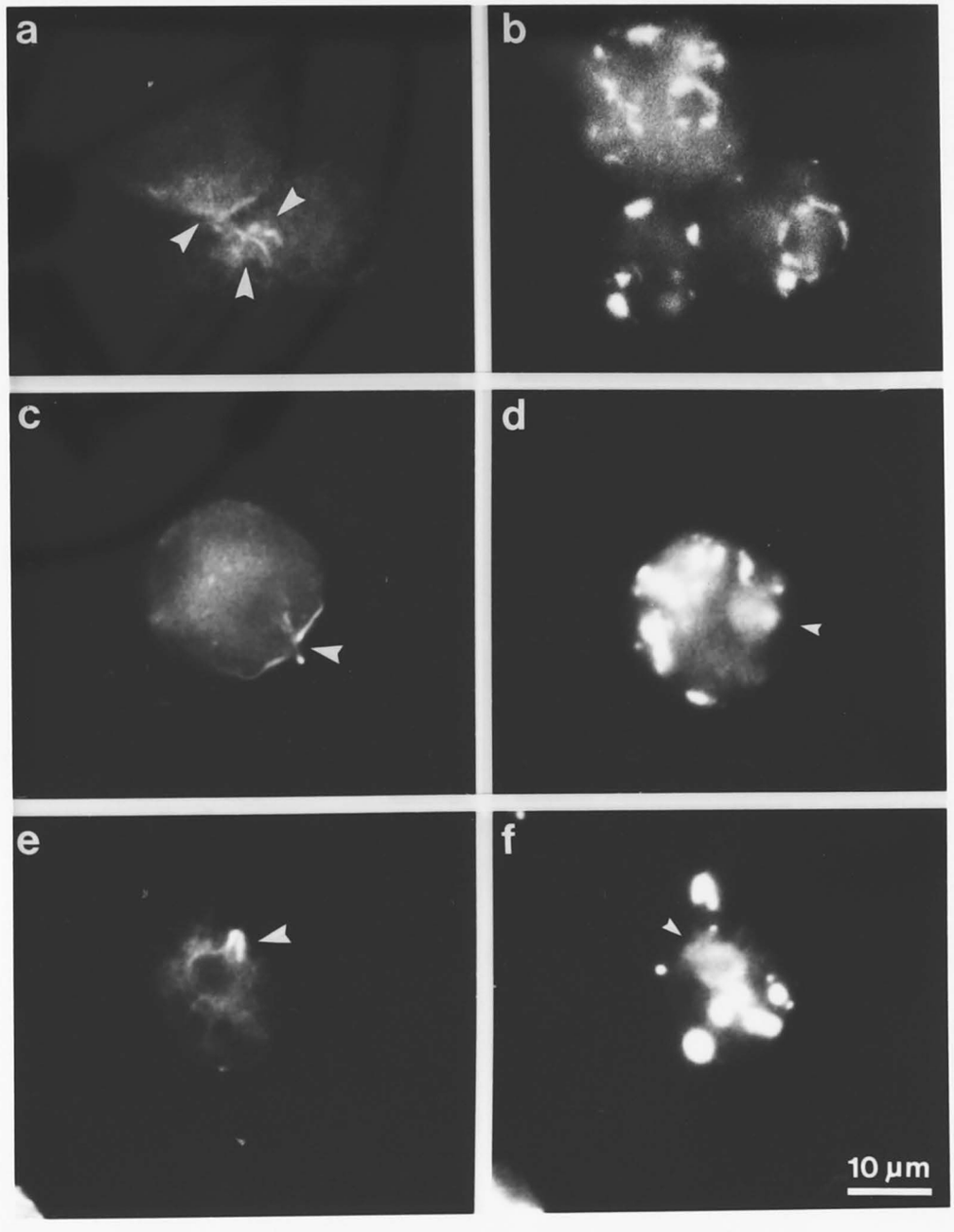


Fig. 3.1.17. Anti acetylated-tubulin and anti centrin antibody staining of the G2-arresting mutant cells, showing that the basal bodies in the arrested cells were not duplicated. (a), anti acetylated-tubulin staining of three *cdM-1* cells each with one pair of basal bodies (arrows); (b), DAPI staining of the cells in (a), showing the prominent chloroplast nucleoides; (c), anti acetylated tubulin antibody staining of an arrested *cdM-2* cell with one pair of basal bodies (arrow). The four cruciate flagellar roots are seen radiating from this single focal point (cf Fig. 2.1.2); (d), DAPI staining of the cell in (c), showing the position of the nucleus; (e), anti centrin staining of an arrested *cdM-2* cell. The centrin had not duplicated in the arrested cell, the two parallel connectors that extend from the nucleus are clearly from their close positioning still attached to a single basal body (arrow); (f), the cell in (e) stained with DAPI showing the position of the nucleus.

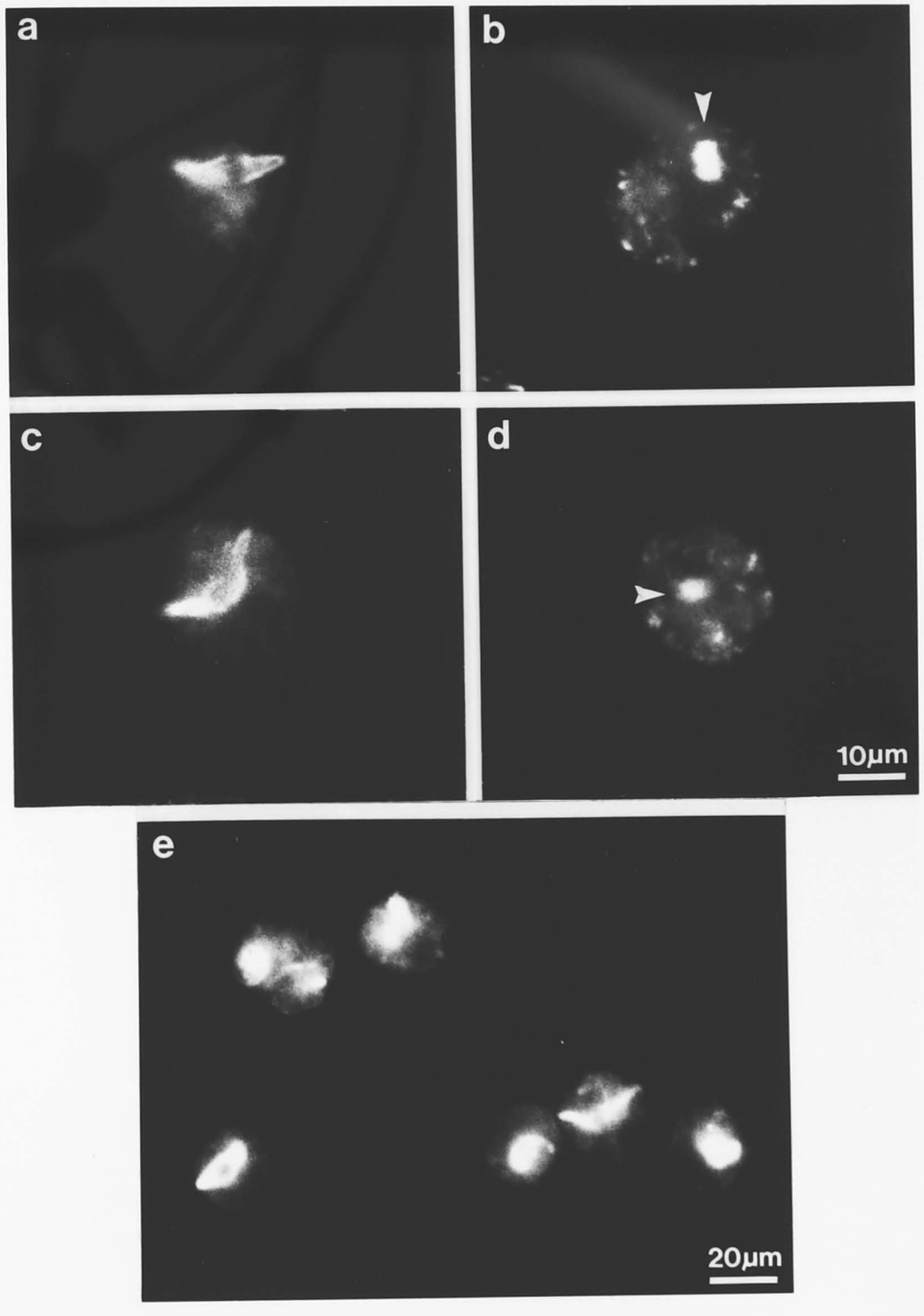


Fig. 3.1.18. Anti β-tubulin antibody staining of arrested *met-1* cells cultured at 33°C for 24 h. (a), (c), two anti β-tubulin antibody stained cells that were arrested at metaphase at 33°C, showing a typical metaphase spindle in each cell; (b), (d), DAPI staining of the cells shown in (a) and (c), indicating the position of the metaphase chromosomes (arrow) aligned in the metaphase plate of each cell; (e), *met-1* cells stained with anti β-tubulin antibody. The cells were arrested uniformly at 33°C, each cell contains a metaphase spindle (seen from different angles).

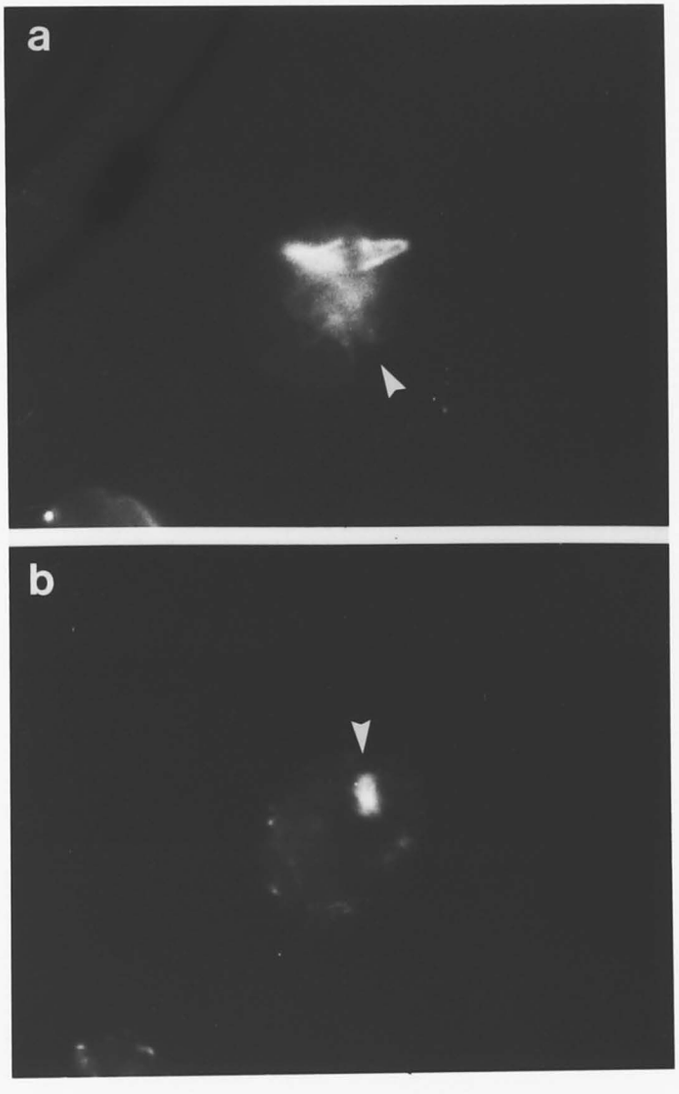


Fig. 3.1.18b. Anti β-tubulin antibody staining of a *met-1* cell arrested at the restrictive temperature, showing that the cell had attempted initiation of cytokinesis. (a), a cell stained with anti β-tubulin antibody, showing a cytoplasmic cleavage furrow (arrow) had initiated in the metaphase arrested cell which contained a fully formed mitotic spindle; (b), DAPI staining of the cell in (a), showing the metaphase array of condensed chromosomes (arrow).

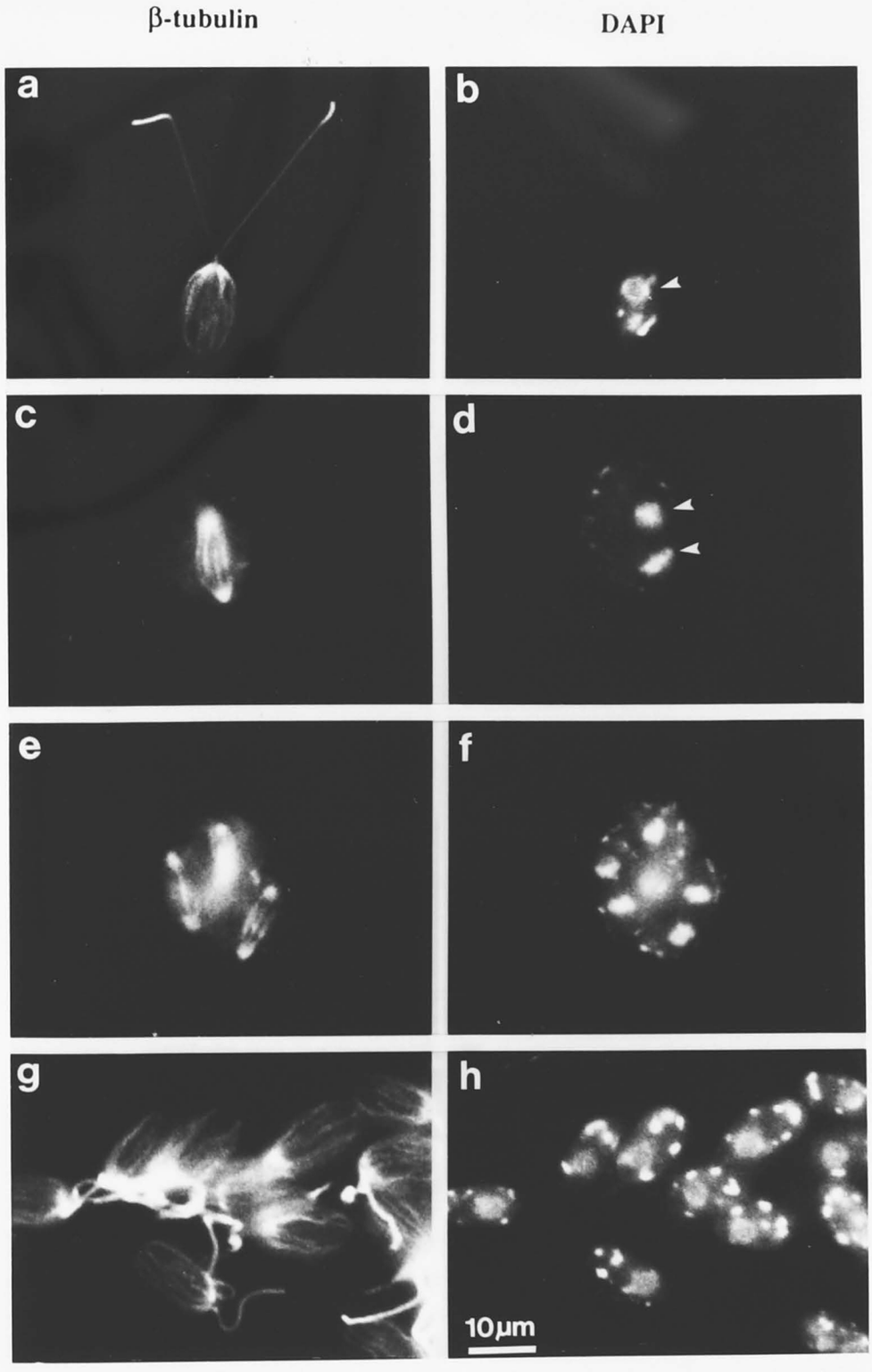


Fig. 3.1.19. Anti β-tubulin antibody staining of *met-1* cells cultured at 21°C, showing that cells of the mutant could undergo normal division at 21°C. *met-1* cells stained with anti β-tubulin antibody, showing respectively; (a), a newly divided cell; (c), a cell in first anaphase; (e), a cell in third anaphase and (g), newly released daughter cells; (b) (d) (f) (h), DAPI staining of the cells. In (b) arrow indicates nucleus, in (d) arrow indicates migrating sets of chromosomes.

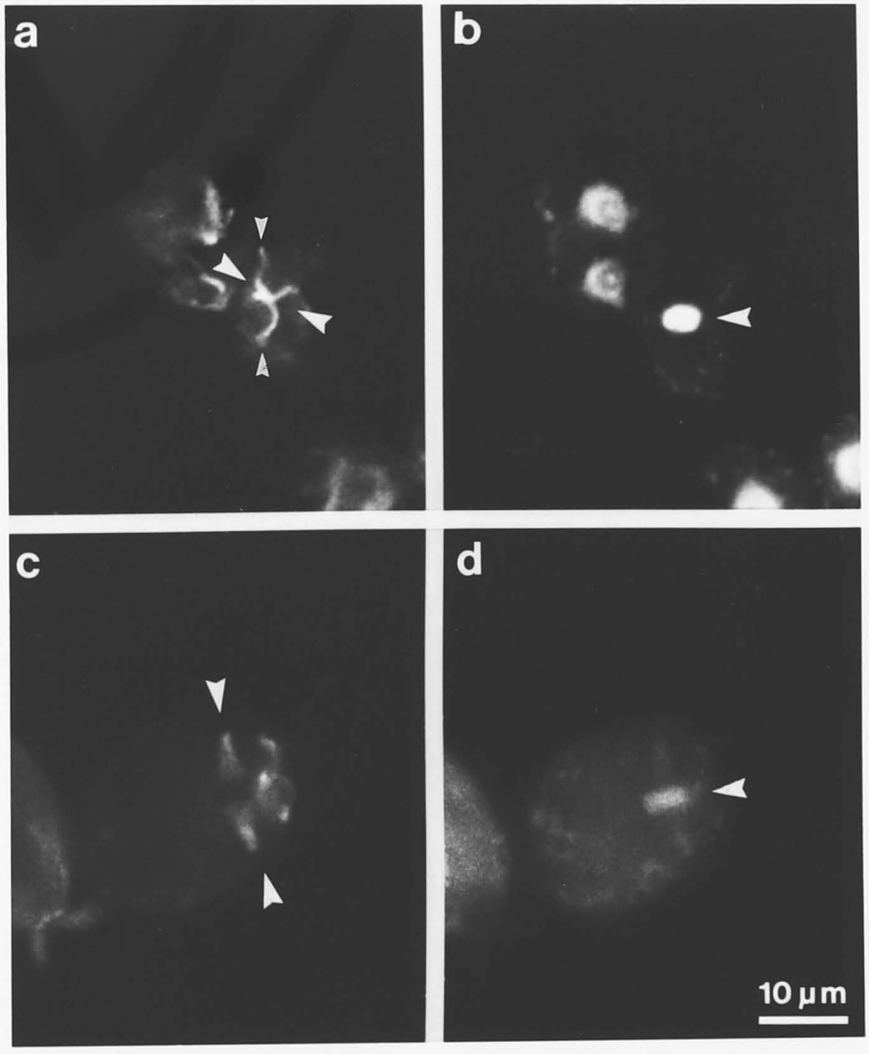


Fig. 3.1.20. Anti acetylated-tubulin antibody staining of *met-1* cells cultured at 21°C (a,b) and 33°C (c,d). (a), anti acetylated-tubulin antibody stained metaphase cells which were cultured at 21°C for 14 h, the antibody stained only the "metaphase band" (between the large arrow heads) and the flagellar roots (small arrow heads) from which it derives; (b), DAPI staining of the cell in (a), showing the metaphase array of chromosomes (arrow); (c), anti acetylated-tubulin antibody stained cells which were arrested at 33°C, the antibody stained both the "metaphase band" and the spindle (arrows); (d), DAPI staining of the cell in (c), showing the metaphase array of chromosomes (arrow).

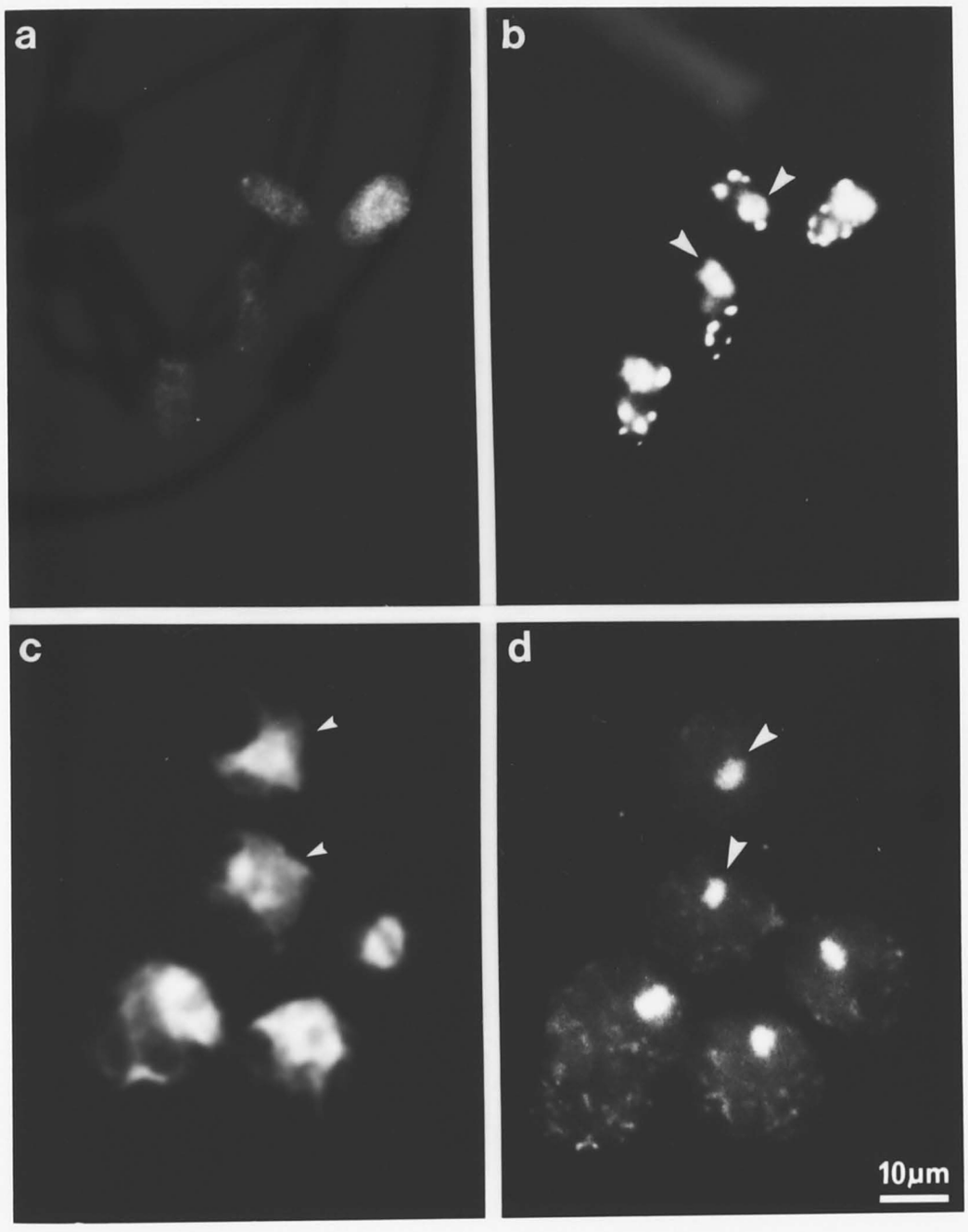
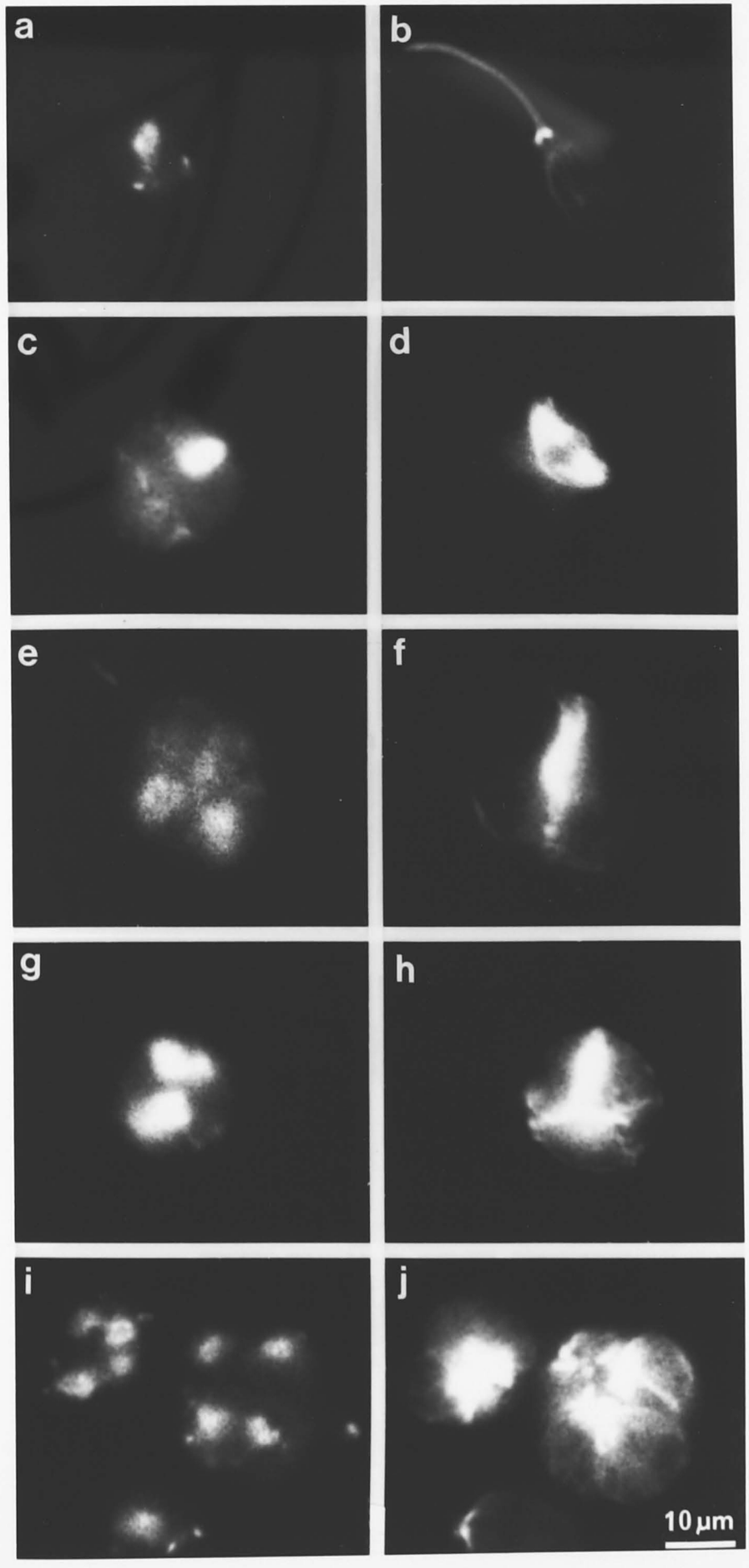


Fig. 3.1.21. Anti MPM-2 antibody staining of synchronous *met-1* cells cultured at 21°C (a, b) and 33°C (c, d) for 24 h. (a), newly-divided *met-1* cells cultured at 21°C stained with anti MPM-2 antibody, No positive staining was observed around the nuclei; (b), DAPI staining of the cells in (a), showing the position of nucleus in each cell (arrows); (c), *met-1* cells arrested at 33°C were stained with anti MPM-2 antibody, abundant MPM-2 proteins were observed around the nucleus of each cell (small arrow heads); (d), DAPI staining of the metaphase array of chromosome (large arrow heads) in cells in (c).

Fig. 3.1. 22. Anti β-tubulin antibody staining of the *cdCK-1* mutant cells that were cultured at 21°C, showing that the mutant cells were able to form a phycoplast and complete cell cycle normally under permissive conditions. (a), DAPI staining of the cell in (b), showing the single interphase nucleus; (b), anti β-tubulin antibody staining of an interphase cell with stained flagella and interphase cortical microtubules; (c), DAPI staining of the cell in (d), showing the array of condensed chromosomes in the cell that was in the first mitosis; (d), anti β-tubulin antibody staining of a cell in the first mitosis, showing that a mitotic spindle had formed in the cell; (e) (g), DAPI staining of cells in (f) (h), showing respectively two and four daughter nuclei formed by one or two preceding mitosis; (f) (h), anti β-tubulin antibody staining of the cells in the first (f) and second (h) mitosis, showing that phycoplasts had formed after each nuclear division; (i), DAPI staining of the cell in (j), showing four nuclei in each newly divided cell; (j), anti β-tubulin antibody staining showing four daughter cells were formed from each mother cell.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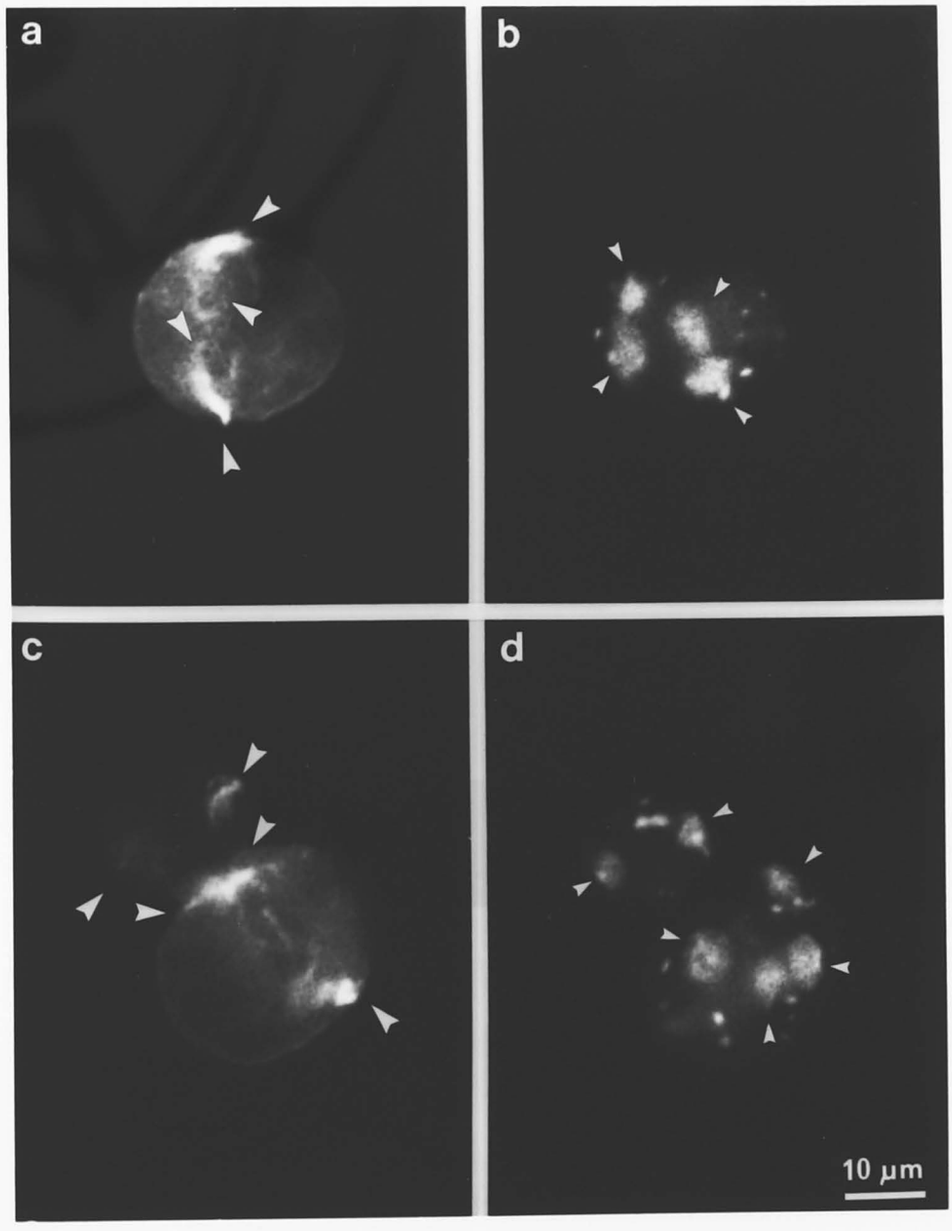


Fig. 3.1.23. Anti acetylated tubulin antibody staining of the arrested *cdCK-1* cells which were cultured at 33°C for 24 h, showing that the cells were arrested as multinucleate with separated basal bodies insert. (a)(c), anti acetylated antibody stained cells; (b)(d), DAPI staining of the cells in (a) and (c), showing the multiple nuclei and their position in the cells. Large arrows, basal bodies; small arrows, nuclei.

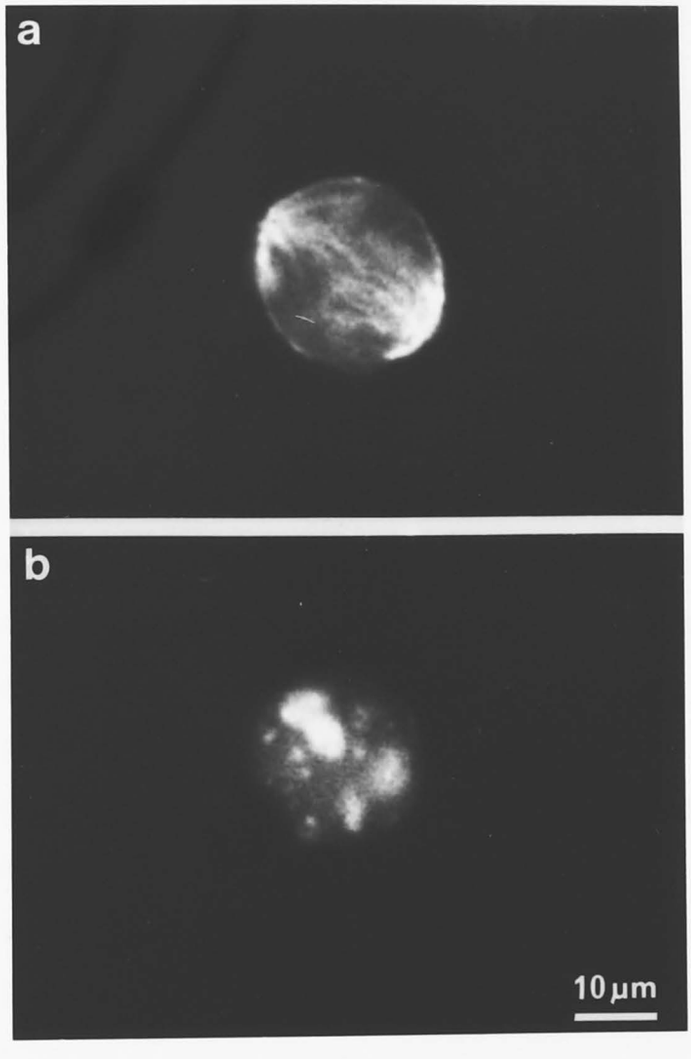


Fig. 3.1.24. Anti β-tubulin antibody staining of arrested *cdCK-1* cells cultured at 33°C for 24 h. (a), anti β-tubulin antibody staining, showing that microtubules were arranged between newly-divided nuclei but no tightly organised phycoplast was formed, in contrast with cells of this mutant dividing successfully at 21°C (seen in Fig. 3.1.22 (f)(h)); (b), DAPI staining of the cell in (a), showing 4 daughter nuclei remaining unseparated by phycoplasts.

Subsection 3.2. Analysis of the G1-Arresting Mutant *cdG1-1*

3.2.1. Introduction

The G1 phase in *Chlamydomonas* occupies a time period that is more than half of the cell cycle. Under conditions allowing rapid growth this extended period allows more than one doubling in cell mass and therefore requires more than one commitment to doubling of cell number for stabilisation of cell size (Donnan et al., 1985). The occurrence of multiple commitments results in cell division by multiple fission, with 2^n daughters being produced per division burst (Jones,1970; Craigie and Cavalier-Smith, 1982). As in yeasts, the first commitment to divide in *Chlamydomonas* marks a transition to a state in which a doubling of nuclear DNA and of cell number will occur even if further net cell growth becomes impossible (John, 1984).

The attainment of commitment can be investigated by testing samples of asynchronous culture transferred to darkness at different times to interrupt growth. Cells that have attained commitment to divide will proceed through rounds of doubling DNA, mitosis and cytokinesis in proportion with their number of commitments even in darkness, but in the absence of growth no new commitment can be undertaken, even if cell size would otherwise allow them (McAteer et al, 1985); therefore the state of commitment at time of sampling can be determined. The facility of being able to take samples from synchronous culture and testing the state of commitment is particularly useful in the analysis of cell cycle mutants. The mutant *cdG1-1* has been identified as arresting in G1 phase of the cell cycle since the nuclear DNA level in the arrested cells remained similar to that of synchronous cells in G1 phase and the cytomorphology of arrested cells revealed a typical interphase appearance, except that flagella had been withdrawn, indicating an early preparation for division (see subsection 3.1). A concern here is whether the mutant cells succeeded in attaining commitment to divide at

restrictive temperature, which could account for flagella withdrawal if it is an early post commitment event.

A further objective in the analysis of the *cdG1-1* mutant was to investigate (1) when during the cell cycle the mutated gene product begins to cause irretrievable abnormality of development at the restrictive temperature and (2) when the mutated gene product completes sufficient of its function for division to occur. These two time points would provide a valuable indication of the possible beginning and completion time between which the gene products performs its essential cell cycle function.

3.2.2. Results

3.2.2.1. Commitment point of the *cdG1-1* mutant

To investigate whether the cells at restrictive temperature attained commitment to divide and if they did at which time, cells from the same parent culture were grown in parallel at both permissive and restrictive temperature from the beginning of the cell cycle (0 h of the illuminated period in a circadian L/D cycle). Samples from both cultures were taken at the times plotted (Fig.3.2.1) and were transferred into darkness at 21°C so that the cells that had attained commitment to divide prior to the time of transfer could complete committed divisions and release daughter cells, but no further commitment could occur after transfer. After 24 h, cell number density of the subcultures was measured with a Coulter Counter to obtain an estimate of the state of commitment at the time of each subculture. Donnan et al. (1985) established that separate commitments, each to a single doubling of cell number, occur in series, therefore the mean first commitment time was taken to be the time when 50% of cells could produce two daughter cells when transferred to darkness and this was indicated by an increase in cell number of 1.5 fold.

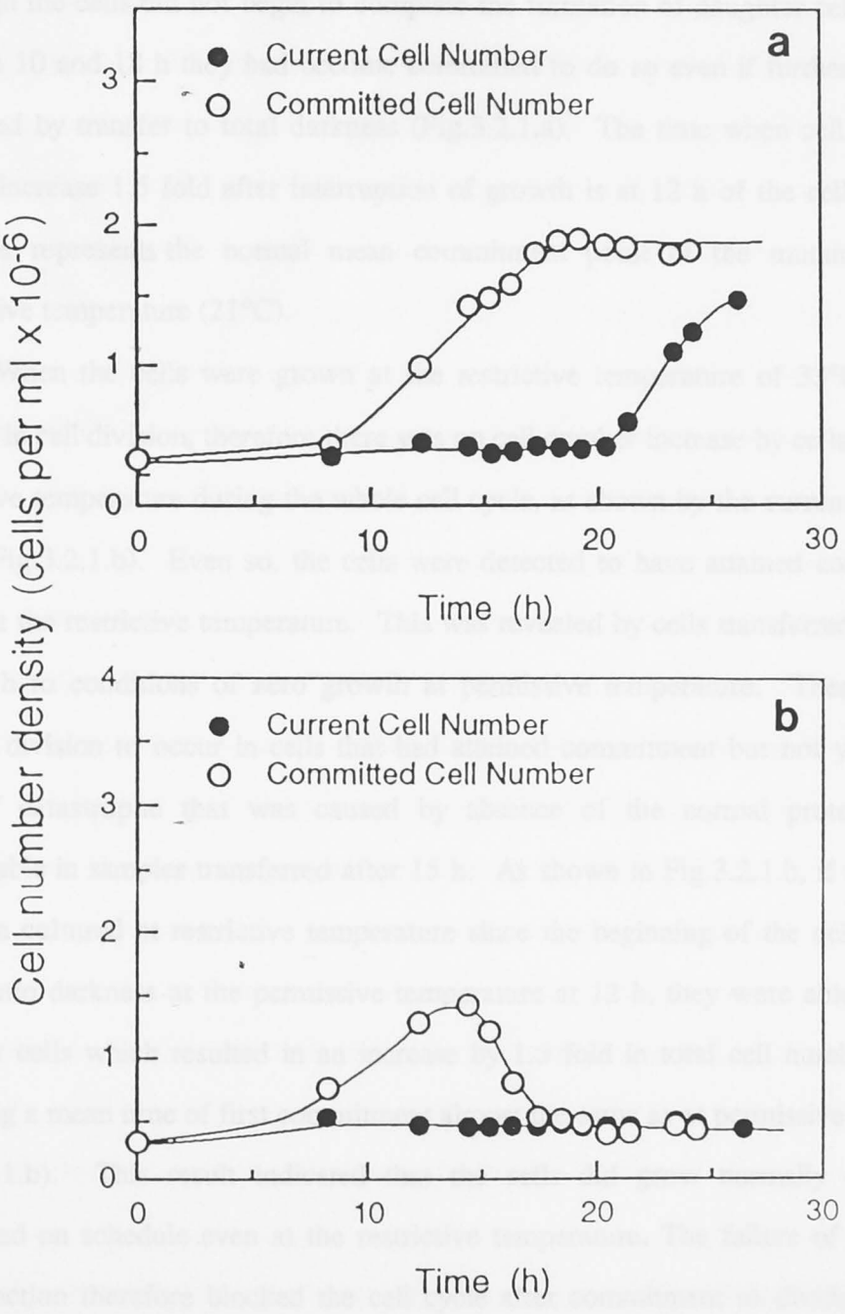


Fig.3.2.1. The G1-arresting mutant *cdG1-1* studied in synchronous culture to determine committed cell number (○) and current cell number (●) at (a) 21°C; (b) 33°C. In both (a) and (b), subcultures transferred to darkness for testing of commitment were held at 21°C after transfer.

As shown in Fig. 3.2.1.a, when cells were grown at 21°C, the current cell number was constant until 20 h when release of daughter cells began and was completed by 24 h. Although the cells did not begin to complete the formation of daughter cells until 20 h, between 10 and 18 h they had become committed to do so even if further growth was prevented by transfer to total darkness (Fig.3.2.1.a). The time when cell number was able to increase 1.5 fold after interruption of growth is at 12 h of the cell cycle which therefore represents the normal mean commitment point of the mutant *cdG1-1* at permissive temperature (21°C).

When the cells were grown at the restrictive temperature of 33°C, they were arrested in cell division, therefore there was no cell number increase by cells remaining at restrictive temperature during the whole cell cycle, as shown by the current cell number curve (Fig.3.2.1.b). Even so, the cells were detected to have attained commitment to divide at the restrictive temperature. This was revealed by cells transferred between 10 and 15 h to conditions of zero growth at permissive temperature. These conditions allowed division to occur in cells that had attained commitment but not yet reached a state of catastrophe that was caused by absence of the normal protein and was irretrievable in samples transferred after 15 h. As shown in Fig.3.2.1.b, if the cells that had been cultured at restrictive temperature since the beginning of the cell cycle were shifted into darkness at the permissive temperature at 12 h, they were able to produce daughter cells which resulted in an increase by 1.5 fold in total cell number therefore indicating a mean time of first commitment almost the same as at permissive temperature (Fig.3.2.1.b). This result indicated that the cells did grow normally and became committed on schedule even at the restrictive temperature. The failure of the mutated gene function therefore blocked the cell cycle after commitment to divide and before detectable nuclear DNA synthesis had occurred. Since flagellar withdrawal occurred at restrictive temperature (Fig. 3.1.7), it seems likely that this is controlled by commitment and occurs separately from the later changes in the cortical microtubules that may be part of mitosis.

3.2.2.2. Execution point of the mutant *cdG1-1*

An experimental strategy was developed for use in yeasts to investigate the time when the function of a gene that has been identified by conditional mutation has been sufficiently completed for the cell cycle to be able to proceed. Shifting cells from permissive to restrictive conditions at different points of the cell cycle identifies a point, termed the execution point, at which restrictive conditions can no longer prevent a mutant cell from successfully completing the cell cycle (Pringle and Hartwell, 1981). This method was applied to identify the execution point of *Chlamydomonas* cdc mutants. To measure the execution point of the mutant *cdG1-1*, synchronous cells were transferred from the permissive temperature (21°C) to the restrictive temperature (33°C) at the times plotted (Fig.3.2.2.). Cell number in each transferred subculture was measured after one cell cycle duration to determine the capacity of the cells to complete the cell cycle under conditions in which the mutated function was not available. The execution point was taken to be the mean time at which cells in a synchronous population acquired the capacity to complete the cell cycle under restrictive conditions. It was measured as the time at which the population achieved a capacity for 50% of the maximum cell number increase; the maximum increase was seen in cells not transferred to restrictive conditions. The execution point for mutant *cdG1-1* was at about 18 h of the cell cycle (Fig. 3.2.2.), which was 6 h after the mean time of first commitment (see in Fig. 3.2.1.a).

3.2.2.3. Catastrophe point of the mutant *cdG1-1*

The execution point provides useful information about the time when the process to which the mutated protein contributes becomes sufficiently completed for division to occur. The execution point, however, does not indicate when function of the mutant protein begins. A new type of experiment has been devised in this research to investigate this aspect and it involves shifting synchronous cells from restrictive temperature (33°C) to permissive temperature (21°C) at different times to observe the time when the mutated gene function begins to cause irretrievable aberrant development.

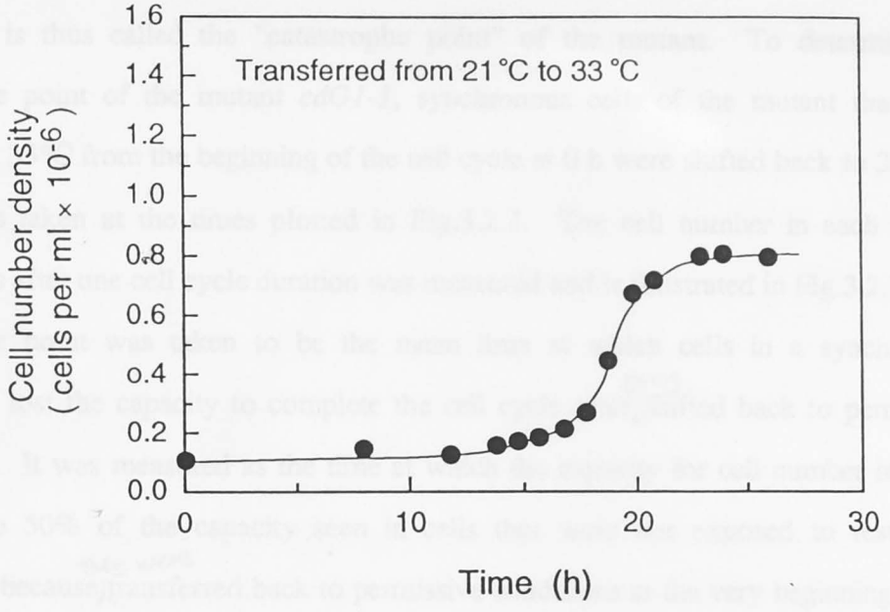


Fig.3.2.2. Final cell numbers of the G1-arresting mutant (*cdG1-1*) seen in samples transferred, at the times plotted, from permissive to restrictive temperature. Samples were transferred from a synchronous culture and maintained in continuing illumination.

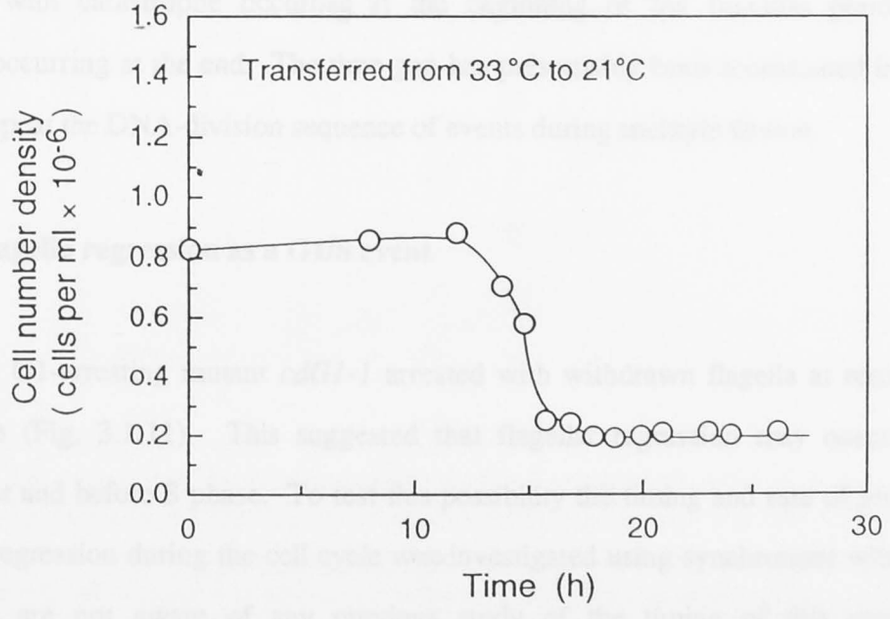


Fig.3.2.3. Final cell numbers of the G1-arresting mutant (*cdG1-1*) seen in samples transferred from restrictive to permissive temperature at the times plotted. Samples were transferred from a synchronous culture and maintained in continuing illumination.

This time is thus called the "catastrophe point" of the mutant. To determine the catastrophe point of the mutant *cdG1-1*, synchronous cells of the mutant that were cultured at 33°C from the beginning of the cell cycle at 0 h were shifted back to 21°C as subcultures taken at the times plotted in Fig.3.2.3. The cell number in each shifted subcultures after one cell cycle duration was measured and is illustrated in Fig.3.2.3. The catastrophe point was taken to be the mean time at which cells in a synchronous population lost the capacity to complete the cell cycle after being shifted back to permissive conditions. It was measured as the time at which the capacity for cell number increase declined to 50% of the capacity seen in cells that were not exposed to restrictive conditions because they were transferred back to permissive conditions at the very beginning of the cell cycle. As shown in Fig. 3.2.3, the mean time that *cdG1-1* mutant cells in synchronous culture lost 50% of the capacity for maximum cell number increase was at 15 h of the cell cycle. The catastrophe point of the mutant *cdG1-1* was thus determined as 15 h, which was 3 h after the mean time of first commitment to division (Fig. 3.2.1. and Fig. 3.2.3). Catastrophe point therefore precedes execution point in a way that is consistent with catastrophe occurring at the beginning of the function period and execution occurring at the end. The time gap has presumably been accentuated in these cells that repeat the DNA-division sequence of events during multiple fission.

2.2.2.4. Flagella regression as a G1/S event

The G1-arresting mutant *cdG1-1* arrested with withdrawn flagella at restrictive temperature (Fig. 3.1.11). This suggested that flagellar regression may occur after commitment and before S phase. To test this possibility the timing and rate of progress of flagella regression during the cell cycle were investigated using synchronous wild type cells. We are not aware of any previous study of the timing of this progress. Synchronous cells were cultured at 21°C and sampled (Fig. 3.2.4) for monitoring the commitment time, flagellar regression and mitosis. As shown in Fig. 3.2.4, cells attained commitment to divide at about 10.2 h. By 13.8 h, 50% of cells in the synchronous population contained residual flagella that had withdrawn at least by half and this is taken

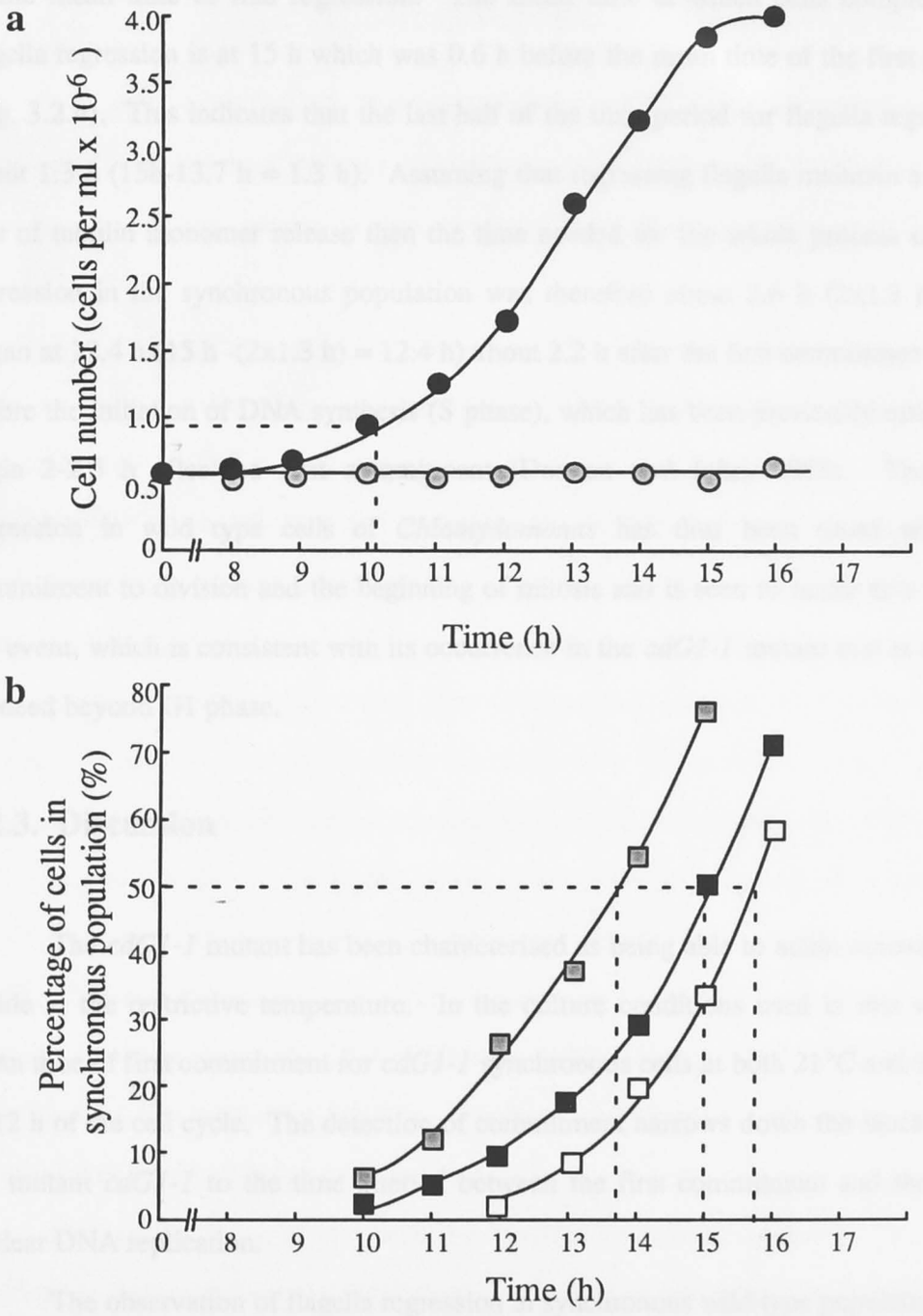


Fig. 3.2.4. Timing of flagella regression during a normal cell cycle of wild type *Chlamydomonas* (*CC-125*$^+$) in synchronous culture at 21 °C. For commitment estimation (a), cells were transferred to darkness and cell number of the transferred subculture was measured after one cell cycle duration on a Coulter counter and plotted at the time transferred. Cells were also fixed by mixing with formaldehyde to a final concentration of 1% for observation of flagella regression by Nomarski microscopy or fixed with 80% ethanol for observation of mitosis by DAPI staining DNA (b). Because fixed flagella are fragile cells were observed immediately and without prior centrifugation. (●), committed cell number; (⊙), current cell number; (▣), percentage of cells with residual flagella of half, or less than full length; (■), percentage of cells that had fully withdrawn flagella; (□), percentage of cells that had initiated first prophase.

as the mean time of mid regression. The mean time at which cells completed their flagella regression is at 15 h which was 0.6 h before the mean time of the first prophase (Fig. 3.2.4). This indicates that the last half of the time period for flagella regression is about 1.3 h (15h-13.7 h = 1.3 h). Assuming that regressing flagella maintain a constant rate of tubulin monomer release then the time needed for the whole process of flagella regression in the synchronous population was therefore about 2.6 h (2x1.3 h), which began at 12.4 h (15 h -(2x1.3 h) = 12.4 h) about 2.2 h after the first commitment and just before the initiation of DNA synthesis (S phase), which has been previously estimated to begin 2-2.5 h after the first commitment (Donnan and John, 1983). The flagella regression in wild type cells of *Chlamydomonas* has thus been timed relative to commitment to division and the beginning of mitosis and is seen to occur as a very late G1 event, which is consistent with its occurrence in the *cdG1-1* mutant that is unable to proceed beyond G1 phase.

3.2.3. Discussion

The *cdG1-1* mutant has been characterised as being able to attain commitment to divide at the restrictive temperature. In the culture conditions used in this work, the mean time of first commitment for *cdG1-1* synchronous cells at both 21°C and 33°C was at 12 h of the cell cycle. The detection of commitment narrows down the block point of the mutant *cdG1-1* to the time interval between the first commitment and the time of nuclear DNA replication.

The observation of flagella regression in synchronous wild type populations in this work has revealed that regression of flagella in *Chlamydomonas* commences as a very late G1 event. The slow disassembly of flagella by depolymerisation at the proximal end was observed to occupy 1.3 h for the first half and presumably close to 2.6. h for the whole process. Its completion occurred about 0.6 h before the mean time of the first prophase (Fig. 3.2.4), thus the basal bodies were available as spindle poles and tubulin monomers were available for spindle formation. This observation has provided the first

evidence that we are aware of for the timing of flagella regression during the cell cycle of this organism.

The arrest point of the G1-arresting mutant *cdG1-1* can be determined as after the time of completion of flagellar regression and just before or at the G1/S transition, since the mutant cells arrested with withdrawn flagella and unduplicated nuclear DNA (see 3.1.2).

The probable time at which the *cdG1-1* gene product functions can be deduced from the results in Fig. 3.2.2 and Fig. 3.2.3. The time at which lack of the normal protein becomes lethal is defined as "catastrophe point" which, in the *cdG1-1* mutant cells, is at 15 h of the cell cycle at 3 h after the first commitment. Before the catastrophe point, the mutated protein was either not present or not active, or the process that the mutated protein is involved in was not yet in progress, therefore when the cells were transferred to permissive temperature, they could continue the cell cycle normally. If the cells of the mutant were shifted after the catastrophe point, the process in which the mutated protein is involved had proceeded abnormally with irretrievable consequences. The timing of 3 h after commitment for the catastrophe point is reasonably coincident with the timing of DNA synthesis at 2-2.5 h after first commitment (Donnan and John, 1983). This coincidence and the arrest of the mutant without having replicated nuclear DNA suggests that it is attempted DNA replication in the absence of the functional *cdG1-1* gene product that within an hour or so becomes catastrophic. Flagella regression is however unaffected and goes to completion.

Conversely the time at which the mutated protein completed sufficient of its function to sustain division is defined as the "execution point" of the mutant. In the *cdG1-1* mutant cells this was attained at 18 h, which was 6 h after the commitment point (Fig. 3.2.2). Before the execution point, cells transferred from 21°C to 33°C were not able to complete their cell cycle because the process in which mutant protein was involved was not sufficiently completed to sustain the nuclear division, therefore no cell number increase occurred. After the execution point, however, cells transferred from 21 °C to 33°C could no longer be blocked by the restrictive condition, thus completion of cell division occurred (Fig.3.2.2.). The time between the "catastrophe point" at 15 h and

the "execution point" at 18 h is the probable time during which the mutated gene product makes its contribution to the cell cycle. During this 3 h period the product may be involved in initiating or sustaining two phases of DNA synthesis as occur in cells producing four daughter cells. The catastrophe point may mark the beginning of the period of function in the first DNA-division sequence and the execution point may mark the completion of function in the final DNA-division sequence.

Subsection 3.3. Analysis of the G2-arresting mutants *cdM-1* and *cdM-2*

3.3.1. Introduction

The G2 phase in *Chlamydomonas* is a very brief period which is difficult to detect even in synchronous populations (Jones, 1970). This raises difficulties for investigation of cell cycle events that occur specifically in G2. Isolation of G2-arresting mutants, however, provides a means to investigate the cell cycle events that occur in the brief G2 period and in addition enables identification of what cell cycle events may be dependent upon completion of G2 phase.

It is well known that depolymerisation of the cortical microtubules in *Chlamydomonas* occurs before mitosis as in most other eukaryotes (Doonan and Grief, 1987). However, it has been difficult to determine how long before mitosis this depolymerisation occurs and in particular no evidence has yet been obtained to indicate whether the depolymerisation of cortical microtubules in *Chlamydomonas* occurs in the brief G2 phase. In addition little is known about what other cell cycle events must be completed before depolymerisation of the cortical microtubules can occur.

Early investigations in our laboratory have shown the presence of a 34 kDa $p34^{cdc2}$-like protein in *Chlamydomonas*, which cross reacts with anti PSTAIR antibody that recognises the perfectly conserved 16 amino acid region present in all cdc2 proteins (John et al., 1989) and has histone H1 kinase activity when purified by $p13^{suc1}$ binding. A homologue of $p13^{suc1}$ has also been detected in *Chlamydomonas* using affinity-purified antibody raised against the fission yeast $p13^{suc1}$ protein (John et al., 1991). The convergence of evidence for presence of the $p34^{cdc2}$-like protein kinase with histone H1 kinase activity and the presence of the $p34^{cdc2}$ binding protein $p13^{suc1}$-like protein suggests that the mechanism that controls the mitotic onset at the G2/M transition in *Chlamydomonas* may be similar to that in other eukaryotes. However, the relationship between these key cell division regulating proteins and the cell cycle of *Chlamydomonas* has not been established.

Investigation of the two G2-arresting mutants designated *cdM-1* and *cdM-2* was directed to the following aspects: (1) the timing of commitment point, execution point and catastrophe point of the two G2-arresting mutants; (2) the relationship of $p34^{cdc2}$ kinase levels and enzyme activity during normal progression through the cell cycle in synchronous populations of wild-type *Chlamydomonas* (*CC-125*$^+$) and in G2-arresting cells; (3) a comparison of the sensitivity of the cells to a microtubule binding drug, oryzalin, was also made between synchronous mutant cells and the wild-type *Chlamydomonas CC-125*$^+$ cells to test whether failure to enter mitosis might be suppressed by an agent that favoured microtubule depolymerisation; (4) in addition observations of the effects of oryzalin on wild type cells were made to test the interrelationship of presence of cytoskeletal elements with catalytic activation of $p34^{cdc2}$ and progress through the cell cycle.

3.3.2. Results

3.3.2.1. Commitment times of mutants *cdM-1* and *cdM-2*

The mutations *cdM-1* and *cdM-2* were identified as causing temperature sensitive G2-arrest. Cells of the two mutants attained commitment to divide at both the permissive and restrictive temperatures (Fig. 3.3.1 and Fig. 3.3.2). Estimation of the mean first commitment times of *cdM-1* and *cdM-2* at both permissive and restrictive temperatures was made by determining when 50% of cells were committed to divide as described in 3.2.2.1. Cells of each mutant were cultured synchronously at 21°C and 33° C. Samples taken from the parallel synchronous populations at various times (see Fig. 3.3.1 for *cdM-1* and Fig. 3.3.2 for *cdM-2*) were transferred into darkness at 21°C to measure commitment to cell division. Cell density in each darkened subculture was measured after one cell cycle duration and is plotted at the time of transfer. Cell numbers in the synchronous culture remained constant until 20 h if mother cells with unreleased daughter cells were counted as one cell, after which daughter cell release occurred (Fig.

3.3.1.a and Fig. 3.3.2.a). The cells had earlier attained the first commitment to divide at about 11 h in *cdM-1* and 9 h in *cdM-2*, at which times cells transferred from 21°C into darkness were able to increase in cell number by 1.5 fold (Fig. 3.3.1.a and Fig. 3.3.2a).

When the *cdM-1* and *cdM-2* cells were grown at 33°C, neither of them were able to complete the cell cycle because of arrest in the G2 phase of the cell cycle, and cell density remained constant (Fig. 3.3.1.b and Fig. 3.3.2.b). However it was detected that commitment was attained by both *cdM-1* or *cdM-2* at the restrictive temperature since transfer to total darkness at the permissive temperature, which prevented further growth and therefore new commitments, allowed division in cells taken in mid cycle that had attained commitment but not yet catastrophe. The mean first commitment point of the mutant cells at the restrictive temperature, revealed by a capacity to increase cell number 1.5 fold in the absence of growth, was at 12 h in *cdM-1* and 12.5 h in *cdM-2* cells (Fig. 3.3.1.b and Fig.3.3.2.b).

3.3.2.2. Catastrophe and execution points of mutants *cdM-1* and *cdM-2*

The time at which absence of the functions encoded by the *cdM-1* and *cdM-2* genes began to cause irreversible obstruction to division was determined as the catastrophe point. Conversely the time at which the encoded functions had been performed sufficiently to allow completion of the current cycle was determined as the execution point. These experimental approaches were as employed for the *cdG1-1* mutant in 3.1.2.2. When synchronous cells of the two mutants were cultured at 33°C and shifted back to 21°C at the times plotted (Fig. 3.3.3), the mean time that cells in the synchronous population lost their capacity to complete their current cell cycle was determined as the catastrophe point of the mutant. The catastrophe point of *cdM-1* was about 12.5 h of the cell cycle (Fig. 3.3.3.a), which was 0.5 h after the commitment point at 33°C (Fig. 3.3.1.b), and the catastrophe point of *cdM-2* was 13 h of the cell cycle (Fig. 3.3.3.b) which was also about 0.5 h after its commitment point at 33°C (Fig. 3.3.2.b). These times are presented in Table 3.3.1. When synchronous cells of *cdM-1* and *cdM-2* were cultured at 21°C and shifted from 21°C to 33°C (Fig. 3.3.3) the mean time

at which the cells attained ability to complete the current cell cycle and increase cell number, was determined as their execution point. The execution point of *cdM-1* was at 14.5 h, about 3.5 h after its commitment point, and the execution point of *cdM-2* was at 15 h, about 6 h after commitment. Again execution points occur a few hours later than catastrophe points, consistent with their marking a completion of function.

Table 3.3.1. The mean commitment, catastrophe and execution points of *cdM-1* and *cdM-2*

	Commitment point		Catastrophe point 33°C→21°C		Execution point 21°C→33°C	
Mutant	at 21°C	at 33°C	time in cycle	h after commitment	time in cycle	h after commitment
cdM-1	11 h				14.5 h	3.5 h
		12 h	12.5 h	0.5 h		
cdM-2	9 h				15 h	6 h
		12.5 h	13 h	0.5 h		

3.3.2.3. Analysis of p34^{cdc2}-like protein levels and protein kinase enzyme activity during a cell cycle of wild type *Chlamydomonas*

The dynamics of change in level and activity of the key cell cycle regulating protein p34^{cdc2} during the *Chlamydomonas* cell cycle is unknown. To analyse whether the *cdM-1* and *cdM-2* mutations, which result in blockage of mitosis, have effects on p34^{cdc2} kinase, it is necessary to understand first how this protein behaves during a normal cell cycle.

p34^{cdc2}-like proteins of *Chlamydomonas* were detected using the anti PSTAIR antibody as a probe. As shown in Fig. 3.3.4, when the anti PSTAIR antibody was used to probe a Western blot of proteins from mitotic cells, four different bands of proteins were detected (Fig.3.3.4. lane 2), which had molecular weights of 32, 34, 36 and 38 kDa. A control analysis of the same extracts shown on lane 1, made by probing with secondary antibody alone, revealed none of the four bands observed on lane 2. Furthermore no positive bands were detected on lane 3 of the same Western blot which was probed with the anti PSTAIR antibody that was pre-competed with the 20 nM PSTAIR peptide (Fig. 3.3.4). This result suggests that the four bands detected by the anti PSTAIR antibody were all PSTAIR containing proteins and therefore *Chlamydomonas* p34^{cdc2}-like proteins.

To analyse levels of the p34^{cdc2}-like proteins in the cell cycle of wild-type *Chlamydomonas*, synchronised cells were cultured in continuous light at 21°C and sampled at different times during the cell cycle, as plotted in Fig. 3.3.5. The levels of p34^{cdc2}-like proteins were determined by detecting the bound anti PSTAIR antibody using ^{125}I anti-rabbit IgG and then quantifying the radioactivity of bound isotope on a PhosphorImager. The levels were low at the beginning and the end of the cell cycle when newly divided cells were in early G1 phase. The total p34^{cdc2}-like proteins reached a maximum level at 14 h of the cell cycle (Fig. 3.3.5) when the percentage of the mitotic cells in the whole population peaked (similar to *met-1* at permissive temperature, Fig. 3.4.3). It was found that the level of band 3 (apparent size 34 kDa) was relatively constant compared with the other PSTAIR protein bands. The levels of band 1 (apparent size 38 kDa), band 2 (apparent size 36 kDa) oscillated as the cell cycle progressed (Fig. 3.3.5). Band 4 (apparent size 32 kDa) also changed in level but to a smaller extent than bands 1 and 2 and reached a peak about 3 h later than the others (Fig. 3.3.5). It is not clear yet whether these four bands of proteins detected by anti PSTAIR antibody are different phosphorylated forms of p34^{cdc2} or different proteins that belong to the cdc2 family. However, all four protein bands were found to be able to bind to the p34^{cdc2} binding protein p13^{suc1} (data not shown).

The $p34^{cdc2}$-like protein kinase activity present during the cell cycle of wild-type *Chlamydomonas* was monitored as histone H1 kinase activity in a purified $p34^{cdc2}$ kinase fraction obtained by affinity purification with $p13^{suc1}$. In this procedure cell extracts were made using NDE buffer (see general methods) from the same samples that were taken for $p34^{cdc2}$ protein level measurement (Fig.3.3.6). The $p34^{cdc2}$ protein kinase was purified from each extract by binding to $p13^{suc1}$-coupled sepharose beads and eluting with free $p13^{suc1}$. Coincident with the oscillation of $p34^{cdc2}$ protein levels described above, activity of the $p34^{cdc2}$-like protein kinase was low from 0 h to 10 h of the cell cycle and reached a maximum level at 14 h, when there were peaks in both mitotic cell number (data not shown) and the $p34^{cdc2}$-like protein level (Fig. 3.3.5). This kinase activity declined sharply with the end of mitotic activity afterwards and returned to the basal level by 24 h of the cell cycle at which time most of the cells in the synchronous population were about to start a new cell cycle (Fig. 3.3.6). The basal level of activity was about 6% of the peak activity, indicating both the very good synchrony obtainable with *Chlamydomonas* cultures and the very tight control exerted over the activity of this key enzyme, which changed sixteen fold in activity per cell fresh weight and therefore close to sixteen fold in specific activity relative to total cell protein, while only changing 2.7 fold in abundance of the protein relative to other proteins (Fig. 3.3.5).

3.3.2.4. Effects of the *cdM-1* and *cdM-2* mutations on the histone H1 kinase activity of $p34^{cdc2}$-like protein

When cells of *cdM-1* and *cdM-2* were incubated at the restrictive temperature of 33°C, they arrested in G2 phase of the cell cycle without depolymerisation of their interphase-like cortical microtubules (although they underwent their cell cycle normally at the permissive temperature of 21°C). It was questioned whether the two mutants were able to activate their $p34^{cdc2}$ kinase under restrictive conditions. Analysis of the $p34^{cdc2}$-like kinase activity in these two mutants was achieved using the same method as for wild-type cells. A very similar oscillation in kinase activity was observed in the cell cycle of these two G2-arresting mutants. When synchronous cells were cultured at the

permissive temperature of 21°C, there was a low level of p34^{cdc2}-like protein kinase activity by 13 h of the cell cycle in synchronous population of both *cdM-1* (Fig. 3.3.7) and *cdM-2* (Fig. 3.3.8). The kinase activity increased about 10 fold to reach the maximum levels in the two mutants at 17 h as the percentage of mitotic cells peaked. There was an obvious decline in the kinase activity when most of the cells in the population completed nuclear division at 24 h (Fig. 3.3.7 and Fig. 3.3.8). When cells of the two mutants were incubated at 33°C, they appeared to grown normally up to their arrest point in G2 phase. The p34^{cdc2} kinase activity in *cdM-1* remained low until at least 13h of the cell cycle then increased slightly by 2 to 2.5 fold by 17 h, which is only a quarter (in *cdM-1*) or one-seventh (in *cdM-2*) of the normal full increase seen at 21°C (Fig. 3.3.7). In *cdM-2* at restrictive temperature the activation of p34^{cdc2} kinase is both weaker and slower becoming significant only at 20 h and reaching only one seventh of 21 °C control levels. In marked contrast with the activity of cells not blocked in division the partially elevated activity in arrested cells of *cdM-1* and *cdM-2* persisted unchanged until the final sample at 24 h (Fig.3.3.7 and Fig. 3.3.8).

3.3.2.5. Effect of the *cdM-1* mutation on the levels of p34^{cdc2}-like protein during the cell cycle of mutant cells

The same method that was used for analysis of the p34^{cdc2} protein levels in wild-type *Chlamydomonas* cells (see 3.3.2.3) was used for the study of *cdM-1* mutant cells. Samples were taken at various times, from a synchronous population that was divided and cultured at both 21°C and 33°C (Fig. 3.3.9). As shown in Fig. 3.3.9, there were four protein bands that were detected by the anti-PSTAIR antibody in the *cdM-1* cells at both permissive and restrictive temperatures. At the permissive temperature, levels of total p34^{cdc2}-like proteins were low from 0 h to 13 h and increased about 3 fold by 17 h (Fig.3.3.9) into the cell cycle as was also observed with wild type cells (Fig. 3.3.5). The level of total p34^{cdc2}-like proteins then decreased as in wild type cells to a low level by 24 h of the cell cycle (Fig. 3.3.9). At the restrictive temperature levels of p34^{cdc2}-like proteins remained more constant, increasing by only about 30% by 17 h, as cells in the

synchronous population became arrested in G2 phase. The slightly increased level of total $p34^{cdc2}$-like proteins in the arrested cells then persisted with only a slight decline by 24 h (Fig. 3.3.9). The small increase in protein level relative to permissive conditions correlated with the smaller increase in activity, which was only threefold compared with eightfold under permissive conditions. The ratios of abundance of the four $p34^{cdc2}$-like protein levels in synchronous *cdM-1* cells cultured at 21°C were similar to those in wild-type cells with band 1 and 2 in particular showing a nearly threefold increase in level at the time when enzyme activity increased eight to nine fold (Fig. 3.3.7). However, when cultured at 33°C, no obvious change was observed in the ratio of abundance of the four $p34^{cdc2}$-like protein levels in synchronous *cdM-1* cells.

3.3.2.6. The effect of the *cdM-1* mutation on the $p34^{cdc2}$ binding protein $p13^{suc1}$

The low $p34^{cdc2}$-like protein kinase activity in the arrested *cdM-1* cells might either be a cause or a consequence of the arrest. A further check was carried out to identify whether the mutation had any effect on the $p34^{cdc2}$ binding protein $p13^{suc1}$ which had been previously detected in wild type *Chlamydomonas* in our laboratory but had not been studied through the cell cycle (John et al., 1991).

To analyse the $p13^{suc1}$ levels during the cell cycle of *cdM-1*, synchronous cells of the mutant were cultured in parallel at both 21°C and 33°C. Samples were taken at the same times as for analysis of the $p34^{cdc2}$-like proteins (Fig.3.3.9). Equal loadings of 50 µg of total protein from cell extracts were separated on a 10-25% gradient acrylamide gel and transferred onto nitrocellulose which was then probed with anti fission yeast $p13^{suc1}$ antibody. The levels of $p13^{suc1}$-like protein were determined by detecting the bound anti $p13^{suc1}$ antibody using ^{125}I labelled anti-rabbit IgG and then quantifying the radioactivity of isotope on the Western blot using a PhosphorImager. It was demonstrated (Fig. 3.3.10) that only a single band of $p13^{suc1}$-like protein was identified in the *cdM-1* cells cultured at both 21°C and 33°C which is consistent with earlier results from wild type cells (John et al., 1991). The levels of $p13^{suc1}$-like protein in the synchronous cells cultured at 21°C were constant throughout the cell cycle (Fig. 3.3.10).

When the mutant cells were cultured at 33°C, the levels of $p13^{suc1}$ also remained constant throughout the cell cycle , but levels were only about two thirds of those in the cells growing normally at 21°C (Fig. 3.3.10).

3.3.2.7. Comparison of the sensitivity of *cdM-1* and wild-type *Chlamydomonas* to the microtubule depolymerising drug oryzalin

Cortical microtubules were maintained in an interphase-like configuration in arrested cells of the G2-arresting mutants *cdM-1* and *cdM-2* (section 3.1.2.4). This observation invited consideration of whether failure to initiate mitosis after the cells reached G2 phase was due to the failure of depolymerisation of the interphase cortical microtubules. Although it is unlikely that the two independently isolated G2-arresting mutants identified the same gene function for depolymerisation of the cortical microtubules an investigation was carried out to confirm whether the *cdM-1* mutation conferred altered microtubule stability and also whether the arrested cells could be induced to depolymerise cortical microtubules and progress into mitosis by the microtubule depolymerising drug oryzalin.

Oryzalin is a widely used dinitroanilin herbicide that binds to tubulin heterodimers in the cytoplasm of plant cells (Morejohn, 1991; Vaughn and Lehnen, 1991). Diverse organisms show different tolerances to the effect of the drug (Upadhyaya and Nooden, 1987; Cleary and Hardham, 1988). Strachan and Hess (1983) reported that [^{14}C] oryzalin binds to *Chlamydomonas* tubulin rapidly and over a wide temperature range. The oryzalin-tubulin complex cannot participate in polymerisation of microtubules while depolymerisation of microtubules can continue from the minus end ("- end"), which eventually results in a complete loss of microtubules and blockage of mitosis. In higher plants this can lead to formation of polyploid nuclei (Morejohn, 1991; Vaughn and Lehnen, 1991). Mutations in tubulin genes that result in altered sensitivity to oryzalin have been documented in *Chlamydomonas* (Schibler and Huang, 1991; James and Lefebvre, 1992; James et al., 1993). If a G2-arresting mutant contained altered microtubules which could not be depolymerised, or a reduced activity of the proteins that

normally trigger depolymerisation in G2, the cells might reveal altered sensitivity to the microtubule drug oryzalin compared with wild-type cells and the genetic defect might be suppressed by presence of oryzalin. A comparison of the sensitivity to oryzalin between *cdM-1* and wild-type cells was made in this work. Synchronous cells of *cdM-1* and wild-type *CC-125*$^{+}$ that were grown at both 21°C and 33°C were treated with a series of different concentrations from 1 μM to 60 μM of oryzalin, at 7 h (late G1) of the cell cycle. The capacity of cells to divide in the presence of oryzalin was quantified by monitoring their ability to produce daughter cells during treatment with oryzalin for one cell cycle duration. It was observed that oryzalin concentrations higher than 30 μM caused an early death at small cell size in both *cdM-1* and wild-type cells. The oryzalin concentrations of 5 μM to 20 μM consistently caused 80-100% inhibition of division while an oryzalin concentration of 2 μM and lower had little effect on the cell division and allowed cell population increase (Fig. 3.3.11 and Fig. 3.3.12). The sensitivity of *cdM-1* cells at 21°C and wild-type cells at both 21°C and 33°C were similar, although the wild-type cells treated with 1-2 μM oryzalin at 33°C revealed a higher number of daughter cells after one cell cycle duration, because of faster growth at 33°C. The concentration of oryzalin that caused 50% inhibition of cell number increase was 2.5 μM in *cdM-1* cells at 21°C and 3 μM in wild type cells at 21°C and 33°C. Immunofluorescence microscopy of both mutant and wild type cells showed that the concentration of oryzalin that caused depolymerisation of cortical microtubules was 5 μ M or higher, but with 10-15 μM of oryzalin treatment, cells of both *cdM-1* and wild-type revealed a more uniform and complete blockage of mitosis.

When wild-type and *cdM-1* cells were cultured at 21°C and treated with a final concentration of 15 μM oryzalin, the cells became arrested with depolymerised cortical microtubules (Fig. 3.3.13) and condensed nuclear DNA. If a longer time of drug treatment was imposed, cells became bigger and looked similar to arrested cell cycle mutants. Nuclei in the arrested cells revealed poor DAPI staining as shown in Fig. 3.3.13. The oryzalin-inhibited cells often contained duplicated and separated basal bodies (Fig. 3.3.14). Wild type cells arrested by 15 μM oryzalin revealed not only duplicated basal bodies and condensed nuclear DNA, but also some cells appeared to

have developed their metaphase microtubule band (MB) without formation of the mitotic spindle (Fig. 3.3.15). Since the metaphase band is formed of residual flagellar roots after the two components of the basal body have moved to the spindle poles, it is therefore revealed that basal body movement and placing are independent of the spindle that is normally present between them. It was further revealed that the metaphase band can be reduplicated without requiring the completion of a preceding mitosis, therefore cells each with two sets of metaphase bands but only one prophase nucleus can be observed in the oryzalin arrested populations (Fig. 3.3.15). After the cells were released from oryzalin, however, the wild type cells remarkably were able to recover from the drug treatment presumably by formation of a spindle between the basal bodies that were linked by metaphase bands (Fig. 3.3.16) and complete cell division to produce daughter cells (Fig. 3.3.16). However, the *cdM-1* cells cultured at restrictive temperature, after 5, 10 and 15 μM oryzalin treatment, arrested with uncondensed nuclear DNA and depolymerised cortical microtubules although in the absence of oryzalin they arrested with persisting cortical microtubules. Therefore the persistence of cortical microtubules in arrested cells was revealed not to be the cause of arrested progress since, when released from oryzalin, the arrested cells without cortical microtubules were unable to recover, neither to reform the interphase cortical microtubules nor to initiate mitosis by 24 h, which is the completion time of a normal cell cycle. This is a point of difference from wild type cells, which were able to recover fully on removal of oryzalin and divide. Rather the mutant cells arrested at 33°C and treated with oryzalin retained their G2-arresting appearance and unduplicated basal bodies, except that the arrested cells also had irreversibly depolymerised cortical microtubules (Fig. 3.3. 17). The result indicates that oryzalin did cause the *cdM-1* mutant cells to depolymerise their cortical microtubules, but could neither prevent from arresting nor rescue the *cdM-1* mutant at the restrictive temperature. The phenotype of *cdM-1* cells at restrictive temperature with oryzalin can be interpreted as showing failure to enter prophase due to lack of full $p34^{cdc2}$ kinase activation and on removal of the inhibitor failure to restore the cortical microtubular cytoskeleton because of the partial establishment of mitosis, perhaps reflected in a partial

but persistent activation of $p34^{cdc2}$ kinase, as seen at 33°C in the absence of oryzalin (Fig. 3.3.7).

3.3.2.8. Effect of the microtubule drug oryzalin on $p34^{cdc2}$ kinase-like activity during the cell cycle of *Chlamydomonas*

Blockage of mitosis by oryzalin in wild-type *Chlamydomonas* resulted in arrest with single nuclei and multiple basal bodies, indicating that basal body duplication and separation can occur without completion of the previous nuclear division. These oryzalin arrested cells, after released from oryzalin containing medium, remarkably proceeded directly into mitosis without requiring further basal body duplication and produced normal daughter cells with one set of flagella and basal body apparatus in each cell (Fig. 3.3.16) although some tetraflagellate cells were occasionally observed. This finding led to further investigations into (1) whether the blockage of mitosis by oryzalin also blocks $p34^{cdc2}$-like protein kinase activity of the cells; and (2) the relationship of cell recovery to $p34^{cdc2}$-like protein kinase activity.

To reveal the effect of oryzalin on the $p34^{cdc2}$ kinase activity, synchronous wild-type *Chlamydomonas* (*CC-125*$^+$) cells were cultured at 21°C from the beginning of a cell cycle. After growing at 21°C for 7 h, the cells were treated with oryzalin by adding 10 mM oryzalin stock dissolved in DMSO directly to the culture, to a final concentration of 15 μM oryzalin. The $p34^{cdc2}$-like protein kinase activity was assayed using histone H1 as a substrate as described in 3.3.2.3. Cells taken from the control synchronous population, not treated with oryzalin, revealed very low $p34^{cdc2}$ kinase activity at 7 h just before they attained the first commitment to divide (Fig. 3.3.18). The activity had increased very little by 12 h then rose to a peak activity by 15 h in uninhibited cells when mitotic activity also peaked. The kinase activity in the control cells then declined slightly by 17 h and returned to the low level by 24 h of the cell cycle. In the presence of oryzalin from 7 h activity also remained low until 12 h and then increased. However, the activity continued to increase after 15 h reaching a maximum by 17 h that was nearly twice the normal level. before declining at 20 h and eventually reached levels as low as in

the control cells by 24 h (Fig. 3.3.18). This result demonstrated that the microtubule depolymerising drug oryzalin disrupted mitosis in *Chlamydomonas* cells, but did not block the oscillation of the $p34^{cdc2}$-like kinase activity, even if execution of mitosis was blocked. There was however an indication that the phase of $p34^{cdc2}$ activation was prolonged in the inhibited cells, which were arrested in a prophase-like nuclear state.

An experiment was carried out to investigate the possible relationship between $p34^{cdc2}$-like kinase activity in the oryzalin arrested cells and the resumption of nuclear division after release from the drug. Synchronous cells cultured at 21°C were treated with a final concentration of 15 μM oryzalin from 7 h and remained in oryzalin containing medium until 24 h. Samples of culture were transferred to oryzalin-free medium for observation of capacity for recovery: at 16 h when the $p34^{cdc2}$ kinase activity had peaked, at 20 h when the $p34^{cdc2}$ kinase activity of the cells had declined to half of the maximum control activity, and at 23 h when the $p34^{cdc2}$ kinase had almost fully inactivated (Fig. 3.3.19). Cell samples taken at these times were freed from oryzalin by washing with oryzalin-free TAPYPP (see 2.2.1) medium and then incubated in this medium for observation of possible recovery using both light and immunofluorescence microscopy. It was observed that the cells released from oryzalin at 16 h of the cell cycle completed cell division by 20 h to 21 h and released daughter cells by 24 h, which was about the same time as the control cells in the parallel synchronous culture without oryzalin. The cells released from oryzalin at 20 h of the cell cycle completed cell division by about 30 h, which was about 9 hours later than the cells released at 16 h, and released daughter cells by 36 h. Furthermore, cells released from oryzalin at 23 h of the cell cycle were not able even to initiate mitosis by 36 h (Fig. 3.3.19). These observations suggested that although oryzalin did not affect the oscillation of $p34^{cdc2}$ kinase activity in the oryzalin treated cells, the decline in $p34^{cdc2}$ kinase activity may have been irreversible since the lower levels resulted in slower progress to division and the lowest level in failure to enter mitosis within the sampling period.

3.3.3. Discussion

Two independently isolated mutants *cdM-1* and *cdM-2* arrest in G2 phase when cells are incubated at the restrictive temperature of 33°C. As described in 3.1.2, arrested cells of these two mutants contained 2C nuclear DNA and interphase-like cortical microtubules, but the cells arrested with different mean cell size even though the growth conditions were identical and different pattens of $p34^{cdc2}$-like kinase activation at the restrictive temperature. The *cdM-1* and *cdM-2* mutations therefore identify two different gene functions that are necessary for completion of the G2 phase and for progression into mitosis.

Commitment measurements indicated that both *cdM-1* and *cdM-2* were able to attain commitment to divide at restrictive temperature, the first commitment times of *cdM-1* and *cdM-2* were at about 12 h and 12.5 h. Cells cultured at restrictive temperature also initiated nuclear DNA replication after the attainment of commitment to divide, but were arrested in G2 phase when they reached the points at which the mutated gene functions were defective. The slight difference in commitment time between *cdM-1* and *cdM-2* might be caused by small differences in growth rate, since mutation in some cell cycle genes might at the same time have some effects on growth (Harris,1989). The occurrence of commitment requires concurrent growth and its timing, although stabilised against temperature drop between 30°C and 20°C is not fully stabilised against slow growth when caused by low light (McAteer et al., 1985; John, 1987).

The functional times of mutated gene products in the two G2-arresting mutants have been approximately identified by temperature shifting experiments, as described in 3.2.2.2 and 3.2.2.3. It may be helpful to note that, although the catastrophe points and execution points of *cdM-1* and *cdM-2* differed slightly in time, measured from the beginning of the cell cycle, account should be taken of differences in overall rate of progress through the cell cycle since differences between cultures, perhaps side effects of their genetic differences, could result in differences in the timing of commitment, which starts the DNA-division sequence. The time of an event after commitment is the best

indication of its location in the DNA-division sequence. On this basis *cdM-1* and *cdM-2* were indistinguishable since the catastrophe point of *cdM-1* was at 12.5 h of the cell cycle and that of the *cdM-2* was at 13 h, but in comparison with their commitment points, both of them revealed catastrophe points at 33°C that were 0.5 h after the mean time of first commitment. The close timing of catastrophe after commitment, which occurs within 0.5 h, is unexpected for mutations that allow progress to G2 phase before arresting at entry to mitosis. The early catastrophe, prior to S phase, may indicate that cells begin early preparation for the later mitotic activation of $p34^{cdc2}$ kinase soon after the late G1 commitment/START control point. One possibility, suggested by measurements of the levels of $p34^{cdc2}$-like proteins in *cdM-1*, is that synthesis of adequate levels of $p34^{cdc2}$-like proteins must begin then, as seen in wild type.

The execution point of *cdM-1* at 21°C was at 14.5 h and that of the *cdM*-2 was at 15 h. Judged relative to the preceding first commitment time at 11 h and 9 h respectively there is a much more rapid progress to the execution point in *cdM-1*, which is attained within 3.5 h, than in *cdM-2* where 6 h elapses after commitment, prior to attainment of execution point. The early attainment of execution point may indicate that the mutated gene product may lose its activity only slowly at restrictive temperature.

Since the two mutations affect cell cycle progress at or about the time that activity of the cell cycle control protein $p34^{cdc2}$ is expected to be present, the activity of this enzyme was studied. In earlier work by John, Sek and Lee (1989), a $p34^{cdc2}$-like protein was detected by anti-PSTAIR antibody, and three electrophoretic forms were identified in mitotic cells of *Chlamydomonas*. It was established directly, by $^{32}PO_4$ labelling, that different phosphorylated forms of $p34^{cdc2}$-like proteins were present but possible presence of additional members of the cdc2 family and the particular amino acids that were phosphorylated were not investigated (John et al., 1989). In work described in this thesis, however, an additional fourth band of $p34^{cdc2}$-like protein was identified in wild-type *Chlamydomonas* extracts and also in the two mutants, *cdM-1* and *cdM-2*. Detection of four $p34^{cdc2}$-like protein bands instead of three might be as a result of better resolution from gradient gel electrophoresis, and in particular bands labelled 3 and 4 here, may have run as a single band in the earlier study. The molecular weights of

the four resolved bands of p34^{cdc2}-like protein were 38-, 36-, 34- and 32-kDa, and were consistent in wild type cells and in all mutants that were investigated. Levels of total p34^{cdc2}-like proteins in wild type synchronous populations oscillated during the cell cycle and peaked during mitosis. Of the four bands detected in synchronous wild-type cells, the 34-kDa band revealed relatively constant levels during the cell cycle while the levels of the others varied coincident with the phases of cell cycle. There is a possibility that the oscillation of p34^{cdc2}-like proteins might have been caused by the stop and start of growth in dark and light that was used to induce synchrony prior to the sampling period or by the declining growth rate of the cells that were in batch culture during the sampling period, although under continuous illumination (reviewed by John et al., 1981). Environmental influences on tubulin synthesis have been observed during the cell cycle in *Chlamydomonas* (Rollins et al., 1983; Nicholl et al., 1988). However, there is no universal disturbance of enzyme patterns since the RUBISCO large subunit protein in the same samples on the same Western blot revealed that it was not obviously fluctuating (data not shown) and neither was the p13^{suc1}-like protein in synchronous *cdM-1* at 21°C (Fig. 3.3.10). Also, one of the four PSTAIR-containing bands (band 3, of 34 kDa) remained relatively constant during the cell cycle. The molecular weight difference of the four p34^{cdc2}-like proteins ranges from 32 to 38 kDa showing a maximum difference of 6 kDa which might represent a nonphosphorylated and three different phosphorylated forms of the p34^{cdc2} protein kinase. Another possible explanation is that the four protein bands may include cdk2-like protein(s) that function in different stages of the cell cycle in mammalian cells (Pines and Hunter, 1991). The relatively constant level of the 34 kDa band (band 3) in Fig 3.3.5 correlates with claims of constant levels of p34^{cdc2} in the cell cycle of fission yeast (Simanis and Nurse, 1986), budding yeast (Reed et al, 1985) and mammalian cells (Draetta and Beach, 1988). It is striking that slower moving forms appear exactly at times when cells become committed to divide (10 h) and enter mitosis (12 h). The slower migrating forms are most easily explained as phosphorylated forms of p34^{cdc2}-like protein, especially since John, Sek and Lee (1989) found $^{32}PO_4$ in approximately 36 and 38 kDa protein immunoprecipitated with antibody against whole p34^{cdc2}. However, only two phosphorylated forms of p34^{cdc2} would be expected if the

pattern of phosphorylation follows that in other eukaryotic cells. A maximally phosphorylated form would have phosphate at a threonine between 160 and 170 in the molecule together with phosphate at tyrosine 15 and, if plants follow the higher eukaryotes, also at threonine 14. This maximally phosphorylated form would be catalytically inactive. A single phosphorylated form would be catalytically active and would retain phosphate only at threonine 160-170. Thus a non-phosphorylated (inactive form) and a multiply-phosphorylated (inactive, early G2 form) and single phosphorylated (active with respect to mitotic substrates) form are expected. Presence of a fourth band would be consistent with presence of a variant form of the $p34^{cdc2}$ protein or with a previously unknown pattern of phosphorylation. It is not possible at present to discriminate between these possibilities. It is relevant that the control of $p34^{cdc2}$ activity in the cell cycle of *Chlamydomonas* is necessarily more complex than in eukaryotes that do not divide by multiple fission. In the alga multiple fission requires the enzyme to be reactivated in a catalytic form that promotes the G1/S transition after the first mitosis has been completed but within the current cell cycle. The rapid alternation of DNA synthesis and mitotic phases (Coleman, 1982) requires a proportionately rapid alternation of catalytic forms of $p34^{cdc2}$. It was speculated (John, 1984) that the multiple fission form of cell cycle could have arisen if $p34^{cdc2}$ repeated its G1/S function, and remarkably it has been demonstrated that certain *cdc2* mutations in *S. pombe* can be induced to repeat in this way. However the *S. pombe* cells thereafter remain diploid (Broek et al., 1991). The way in which alternative catalytic forms of $p34^{cdc2}$, or members of that family, drive an alternation of S and mitotic phases in *Chlamydomonas* remains unclear. The brief time available between alternations (one to two hours) could make it difficult to change the cyclin populations of the cell between G1 and G2 forms, however this is not impossible since a change from presence of G1 cyclins to G2 cyclins occurs within 2 h in the budding yeast cell cycle (review by Reed, 1991). Alternatively the possibility that a form of $p34^{cdc2}$ that is permanently in G1/S or G2/M mode but held inactive in some way during phases when it is not required is an interesting area for future study.

In wild-type synchronous cells, the activity of the p34^{cdc2}-like protein kinase, as in other organisms (Draetta and Beach, 1989; Gould and Nurse, 1989), oscillated with the cell cycle and reached a maximum level when most cells were in mitosis.

The *cdM-1* and *cdM-2* arrest phenotypes are somewhat similar with respect to p34^{cdc2} kinase-like activity to the G2-arresting mutant *nimA5* in *Aspergillus,* which arrests with duplicated nuclear DNA, interphase cortical microtubules (Oakley and Morris, 1983) and fully activated p34^{cdc2} kinase activity (Osmani, 1991). However, both *cdM-1* and *cdM-2* mutants contained unduplicated basal bodies and only partial p34^{cdc2}-like kinase activity after they became fully arrested at restrictive temperature whereas *nimA5* in *Aspergillus* has duplicated spindle pole bodies and fully activated p34^{cdc2} (Osmani et al., 1991). It is not yet clear whether the arrest of *Chlamydomonas* mutants *cdM-1* and *cdM-2* is caused by the partial completion of processes which fully activate the p34^{cdc2} kinase. It is alternatively possible that the failure in fully activating the p34^{cdc2} is a consequence of the blockage of G2 events that precede p34^{cdc2} activation, although it is not clear what these might be.

Previous investigation (James and Lefebvre, 1992) showed that *Chlamydomonas* exhibits extreme sensitivity to microtubule drugs such as the phosphoric amide herbicide amiprophos-methyl (APM) and the dinitroaniline herbicide oryzalin. On agar medium, wild-type cells die at 5 μM oryzalin (James and Lefebvre, 1992). In this work, cells recovered from 15 μM oryzalin if the cells were released from the drug within a time equal to one cell cycle even though the oryzalin caused depolymerisation of the cortical microtubules and blocked nuclear division.

It has been found that the p34^{cdc2} kinase activity in the oryzalin treated cells oscillates like the untreated cells, except that the peak of the p34^{cdc2} kinase activity in the oryzalin treated cells continues to increase for longer and reaches higher levels although no spindle is present. This evidence supports an independent relationship between spindle formation/metaphase-anaphase transition and the activation/inactivation of the p34^{cdc2} kinase during the cell cycle of *Chlamydomonas*. The independence of p34^{cdc2} kinase activation/inactivation upon the spindle formation and passage though metaphase-anaphase transition, reported here, is the first observation of this phenomenon

in *Chlamydomonas*. A similar situation has been documented in budding yeast (Murray, 1992). However, the tight coupling of p34^{cdc2} kinase activity seen here with ability of cells to recover from microtubule depolymerising drug has not been reported in other organisms.

The ability of cells to recover from oryzalin treatment seems to be related to the level of p34^{cdc2} kinase activity that the oryzalin treated cells have at the time when they are released from the drug. Wild-type cells that hold a maximum level of p34^{cdc2} kinase activity can form a mitotic spindle immediately after being released from the drug treatment while the cells with reduced p34^{cdc2} kinase activity were delayed in the recovery after released from the drug treatment. The *cdM-1* mutant cells were unable to be rescued by treating the cells with oryzalin to depolymerise the cortical microtubules that otherwise persist at restrictive temperature. This failure of rescue points to another defect as the cause of arrest and the failure to normally activate p34^{cdc2}-like kinase is a possible candidate.

The finding that the two independent mutants both contained an interphase-like cortical microtubule cytoskeleton, indicates that the cell cycle events identified by these two mutants occurred before the depolymerisation of cortical microtubules. These mutants provide the first evidence to suggest that depolymerisation of cortical microtubules in *Chlamydomonas* does occur during the brief G2 period of the cell cycle. Since the G2 phase in *Chlamydomonas* is very brief, the interval between the *cdM-1* or *cdM-2* gene product becoming functional and the initiation of mitosis may be even shorter. The recognition of a mitosis associated change in the cortical cytoskeleton was supported by the observation that regression of flagella and depolymerisation of interphase cortical microtubules proceeded separately, with the flagella regression occurring as a G1/S event (see 3.2.2.4) and cortical microtubule depolymerisation as a G2/M event. It has been described in section 3.2 that flagella regression is initiated prior to S phase and is completed before the first prophase in wild type cells (see 3.2.2.4). Conversely the depolymerisation of cortical microtubules is a late event that requires proper initiation of mitosis, which does not occur in either *cdM-1* or *cdM-2* mutants which activate p34^{cdc2}

kinase to only one quarter or one seventh of the normal mitotic level respectively (Fig. 3.3.7 and Fig. 3.3.8) and fail to initiate chromosome condensation.

That the *cdM-1* gene may not directly function in the event of depolymerisation of the cortical microtubules was supported by the result from the experiment of oryzalin treatment. When *cdM-1* mutant cells were treated with 15 μM oryzalin at the restrictive temperature, the cortical microtubules were depolymerised in the arrested cells but no chromosome condensation was observed. The control wild-type cells recovered from the drug treatment and initiated mitosis immediately after oryzalin was washed out. However, the treated mutant cells remained in the G2-arresting state after release from the oryzalin treatment. The result suggests that enabling the mutant cells to depolymerise their microtubules by oryzalin treatment does not help the cells to correct the defect caused by the mutated gene function. This may be because the mutation occurred in a gene that functions at the G2/M transition while depolymerisation of the interphase cortical microtubules is, in regulatory terms, part of the mitotic initiation events that are downstream of normal *cdM-1* and *cdM-2* function.

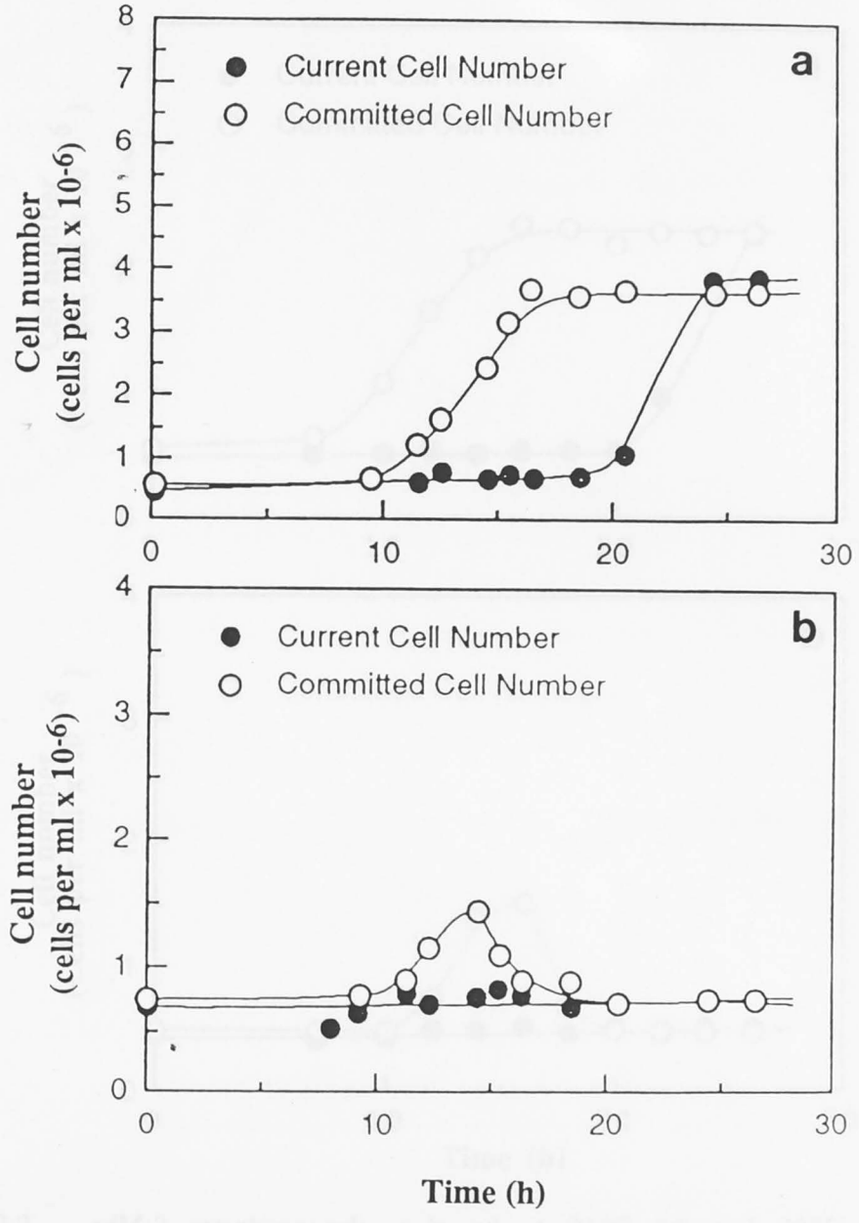


Fig. 3.3.1. *cdM-1* synchronously cultured at 21°C (a) and 33°C (b) for determination of current cell number and committed cell number. For measurement of the current cell number (closed circles), samples were taken at various times and fixed with final concentration of 1% formaldehyde. After dilution 1:50 in 0.9% saline, cell numbers were measured with a Coulter counter and are plotted at the times sampled. For measurement of commitment to division (open circles), samples from both 21°C and 33°C cultures were transferred to total darkness at 21°C with continued aeration provided by orbital shaking until all committed divisions were complete, then cell number was measured with a Coulter counter. Committed cell numbers are plotted at the time growth was arrested by transfer to darkness and therefore any further commitments prevented.

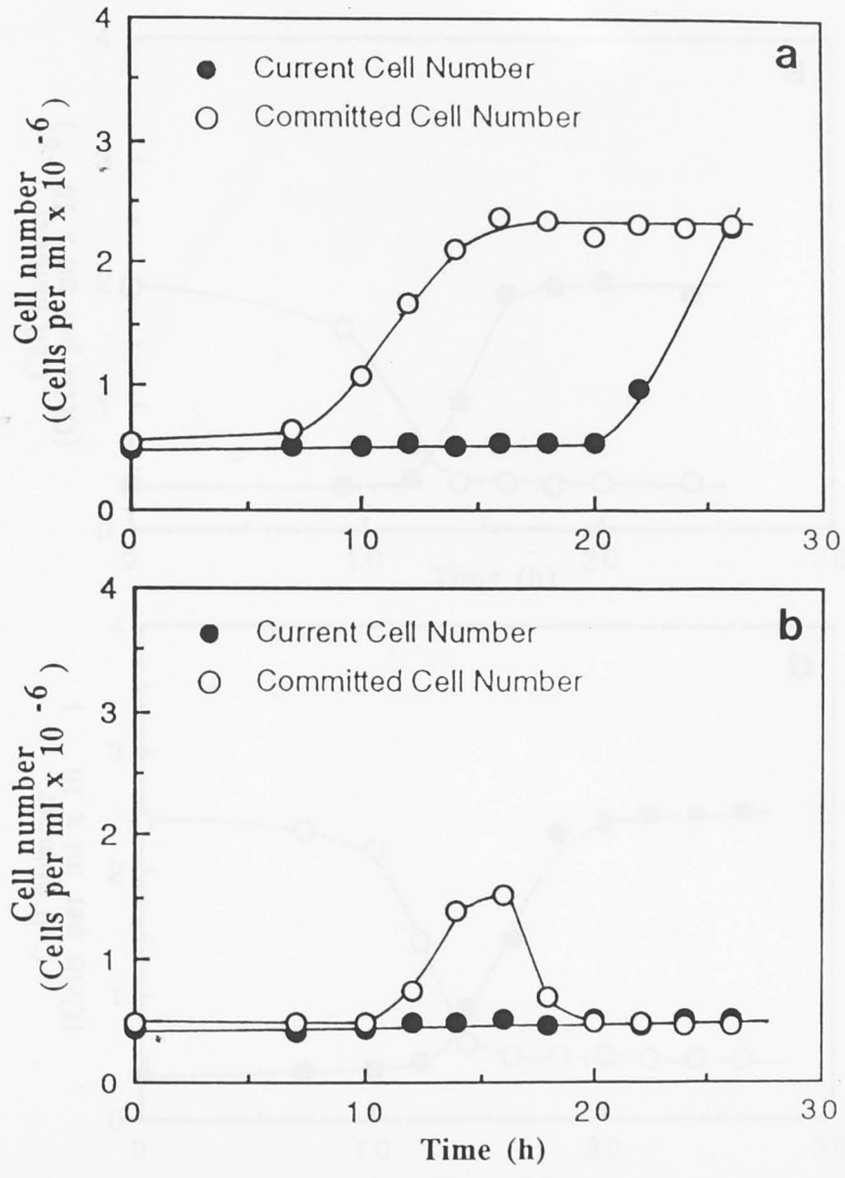


Fig. 3.3.2. *cdM-2* synchronously cultured at 21°C (a) and 33°C (b) for determination of current cell number and committed cell number. For measurement of the current cell number (closed circles), samples were taken at various times and fixed with final concentration of 1% formaldehyde. After dilution 1:50 in 0.9% saline, cell numbers were measured with a Coulter counter and are plotted at the times sampled. For measurement of commitment to division (open circles), samples from both 21°C and 33°C cultures were transferred to total darkness at 21°C with continued aeration provided by orbital shaking until all committed divisions were complete then, cell number was measured with a Coulter counter. Committed cell numbers are plotted at the time growth was arrested by transfer to darkness and therefore any further commitments prevented.

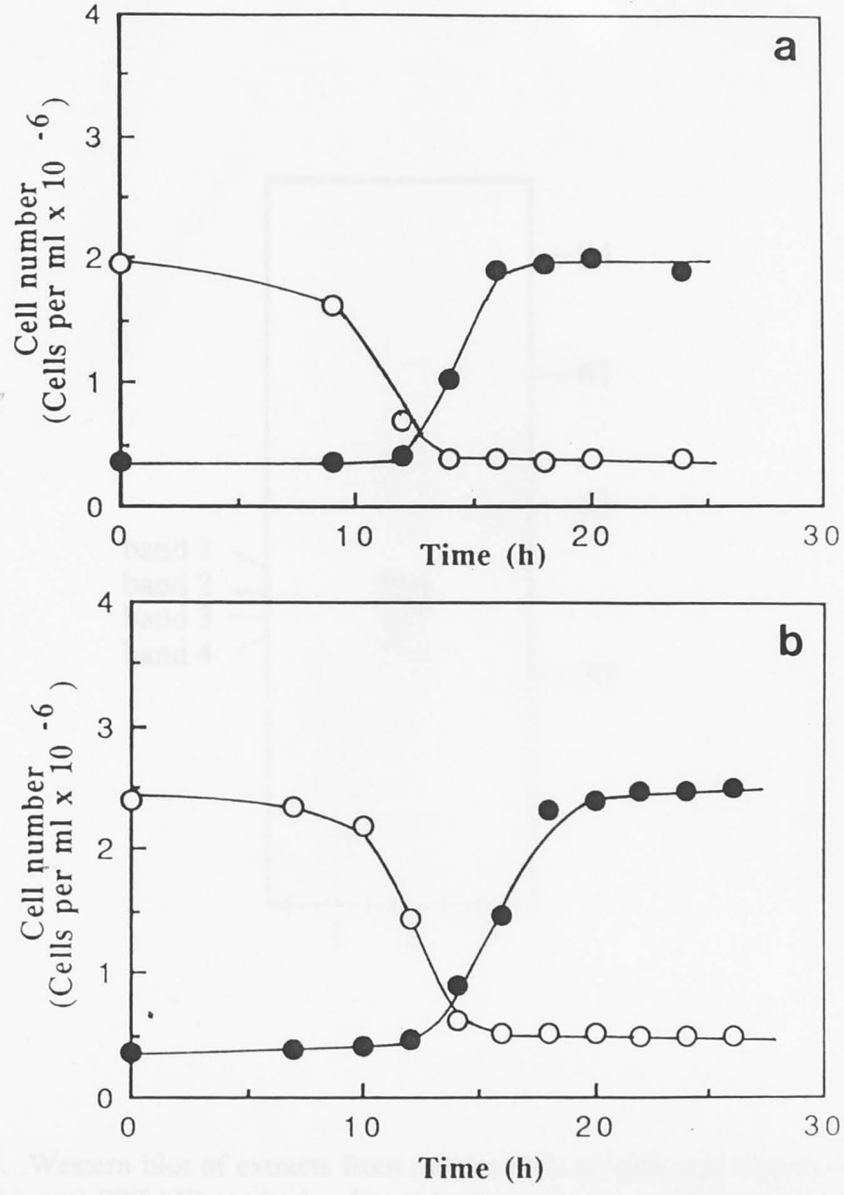


Fig. 3.3.3. Cell number density in (a) *cdM-1* and (b) *cdM-2* cultures, shifted at the beginning of the cell cycle from 21°C to 33°C (closed circles) and from 33°C to 21° C (open circles). Synchronous cells of *cdM-1* and *cdM-2* were cultured in parallel at 21°C and 33°C from the beginning of the cell cycle at 0 h. For determining the catastrophe point, samples taken at the times plotted were transferred from 33°C to 21°C, then after one cell cycle duration (at 48 h) cell number in each transferred subculture was measured and is plotted at the time transferred (open circles). For determining the execution point, samples taken at times plotted were transferred from 21°C to 33°C, then after one cell cycle duration (at 48 h) cell number in each transferred subculture was measured and is plotted at the time transferred (closed circles). Transferred samples, in both (a) and (b), were in continuing illumination.

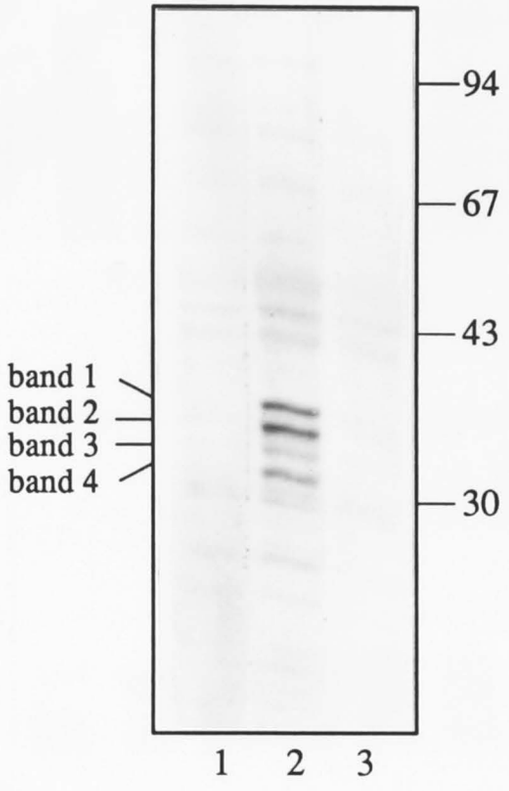


Fig. 3.3.4. Western blot of extracts from mitotic cells of wild type *Chlamydomonas* probed with anti-PSTAIR antibody. Equal loading of total cell proteins extracted in RIPA buffer were run in adjacent lanes on a 10-15% linear gradient acrylamide gel and transferred onto nitrocellulose. The blot was probed with affinity purified polyclonal anti-EGVPSTAIREISLLKE antibody. Bound antibody was detected by goat anti-rabbit-alkaline-phosphatase-conjugated second antibody. Strips were incubated (1) without first antibody, (2) with uncompeted first antibody, (3) with first antibody precompeted with 20 nM EGV peptide EGVPSTAIREISLLKE. Molecular size standards are indicated, units kDa.

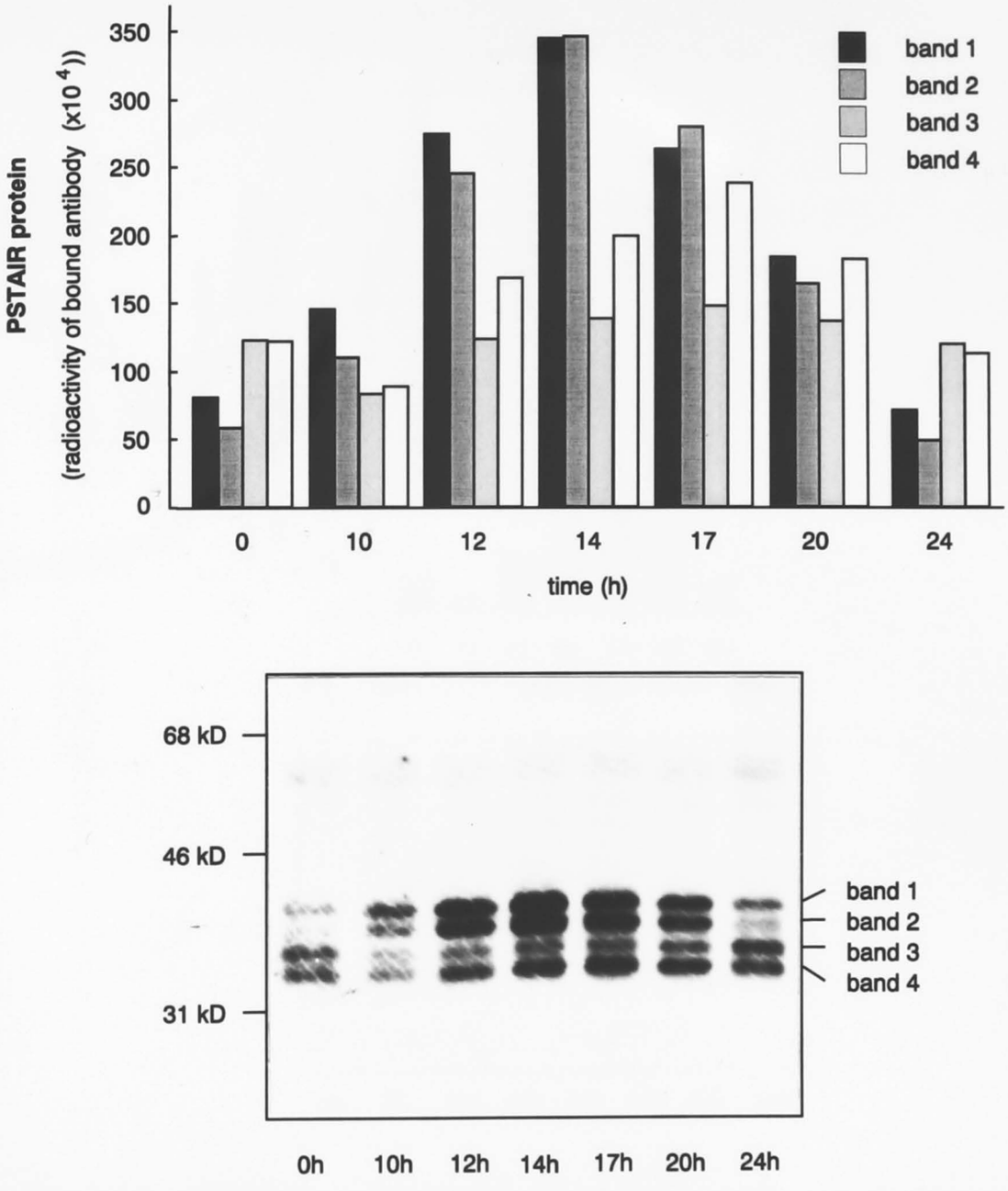


Fig. 3.3.5. Levels of $p34^{cdc2}$-like proteins during a cell cycle of wild-type *Chlamydomonas* (*CC-125*$^{+}$). Previously synchronised cells were cultured in continuous light at 21°C from the beginning of the cell cycle. Samples were taken at various times during the cell cycle. Samples of cells were ground in liquid nitrogen and extracted with RIPA buffer (see general methods 2.2.15). Equal (50 μg) loadings of total protein were separated on a 10-15% linear gradient SDS-acrylamide gel and transferred onto nitrocellulose. The Western blot was probed with anti-EGVPSTAIREISLLKE (PSTAIR) antibody and the bound antibody was detected by ^{125}I anti-rabbit IgG. The image shown at the bottom of the figure, was obtained by exposure of the nitrocellulose on Kodak X-omat film. Four protein bands were detected by the antibody as shown in the blot. Levels of the four bands of $p34^{cdc2}$-like protein were determined by quantification of the bound isotope on a PhosphorImager.

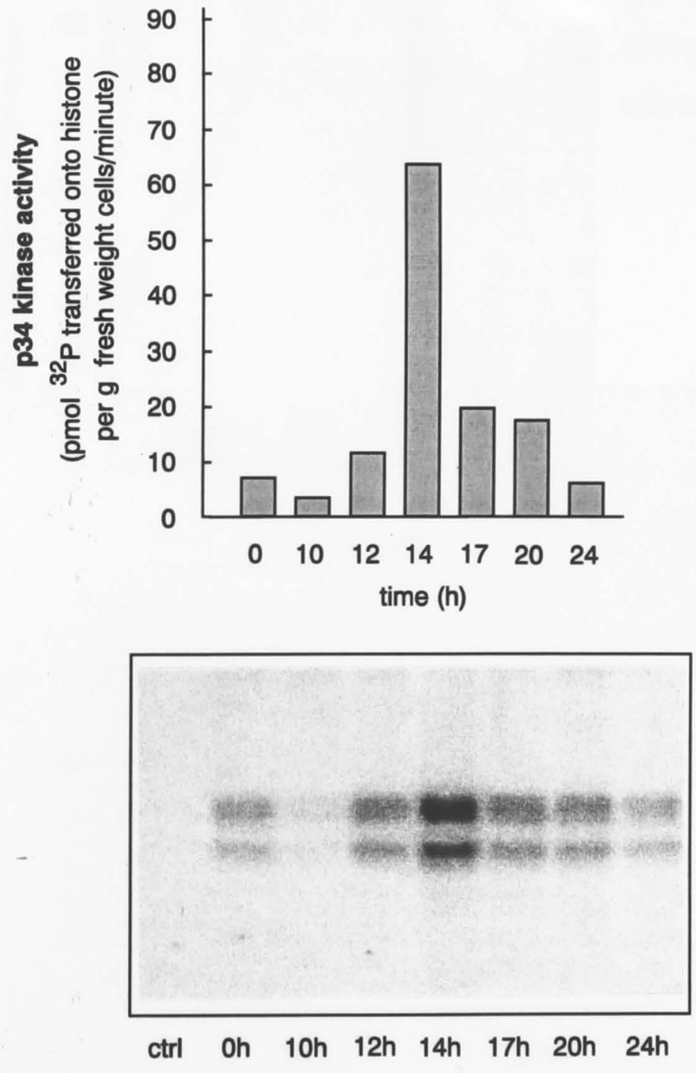


Fig. 3.3.6. $p34^{cdc2}$-like protein kinase activity during a cell cycle of wild-type *Chlamydomonas* (*CC-125*$^{+}$). Previously synchronised cells were cultured in continuous light at 21°C and sampled at various times during the cell cycle. 0.01 g samples of cells ground in liquid nitrogen were extracted with NDE buffer (see general method 2.2.19). The $p34^{cdc2}$-like protein kinase was purified from each sample by binding to 20 µl of $p13^{suc1}$ beads, two washes with detergent buffer and one wash without detergent then eluting with 50 µl of 0.5 mg/ml of free $p13^{suc1}$ solution. The activity of purified $p34^{cdc2}$-like protein kinase was measured using histone H1 as a substrate at 30°C for 5 minutes. The activity is illustrated as the amount of ^{32}P transferred onto histone H1, which was measured by placing 20 µl of reaction mixture on P81 phosphocellulose paper, washing with 75 mM phosphoric acid and then counting in a scintillation counter. The radioactivity of the reaction product, phospho-histone H1, shown at the bottom of the figure, was obtained by separation of 30 µl of reaction mixture on a 12% acrylamide gel and then exposure in a PhosphorImager.

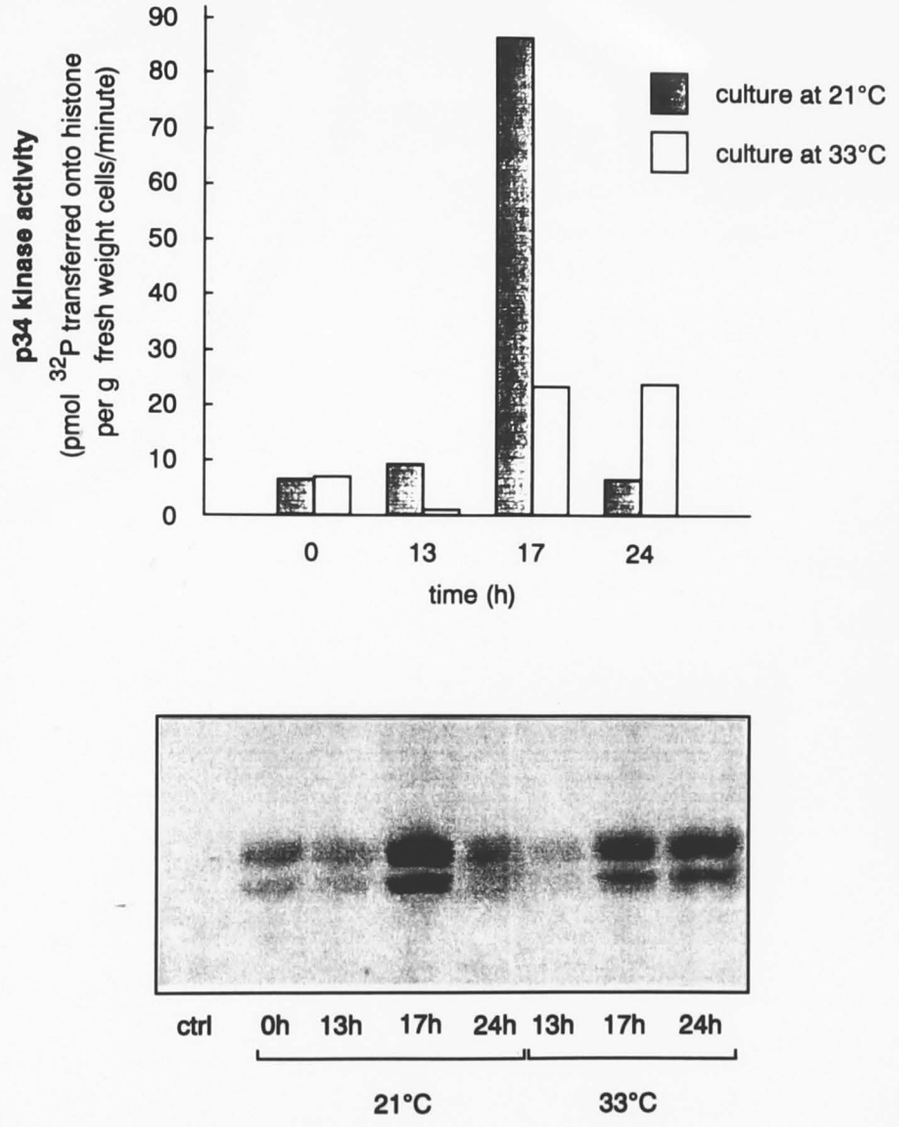


Fig. 3.3.7. Effect of the *cdM-1* mutation on p34^{cdc2}-like kinase activity during the cell cycle. Synchronous cells of *cdM-1* were cultured in parallel at both 21°C and 33° C and sampled from the beginning of growth in newly formed daughter cells. For measurement of p34^{cdc2}-like kinase activity, 0.01 g samples of cells ground in liquid nitrogen were extracted in NDE buffer (see general method 2.2.19). The p34^{cdc2}-like protein kinase was purified from each sample by binding to 20 µl of p13^{suc1} beads, two washes with detergent buffer and one wash without detergent, then eluting with 50 µl of 0.5 mg/ml of free p13^{suc1} solution. The activity of purified p34^{cdc2}-like protein kinase was measured using histone H1, as a substrate at 30°C for 5 minutes. The activity was illustrated as the amount of ^{32}P transferred onto histone H1 which was measured by placing 20 µl of reaction mixture on P81 phosphocellulose paper, washing with 75 mM phosphoric acid and then counting in a scintillation counter. The radioactivity of labelled phospho-histone H1, shown at the bottom of the figure, was obtained by separation of 30 µl of reaction mixture on a 12% acrylamide gel and then exposure in a PhosphoImager.

Fig. 3.3.8. Effect of the *cdM-2* mutation on $p34^{cdc2}$-like kinase activity during the cell cycle. Synchronous cells of *cdM-2* were cultured in parallel at both 21°C and 33 °C and sampled from the beginning of growth in newly formed daughter cells. For measurement of $p34^{cdc2}$-like kinase activity, 0.01 g samples of cells ground in liquid nitrogen were extracted in NDE buffer (see general method 2.2.19). The $p34^{cdc2}$-like protein kinase was purified from each sample by binding to 20 µl of $p13^{suc1}$ coupled beads, two washes with detergent buffer and one wash without detergent, and eluting with 50 µl of 0.5 mg/ml of free $p13^{suc1}$ solution. The activity of purified $p34^{cdc2}$-like protein kinase was measured using histone H1 as a substrate at 30°C for 5 minutes. The activity was determined as the amount of ^{32}P transferred onto histone H1, which was measured by placing 20 µl of reaction mixture on P81 phosphocellulose paper, washing with 75 mM phosphoric acid and then counting in a scintillation counter. The radioactivity of the reaction product, phospho-histone H1, shown at the bottom of the figure, was obtained by separation of 30 µl of reaction mixture on a 12% acrylamide gel and then exposure in a PhosphorImager. The control treatment (ctrl) was an assay performed without adding enzyme.

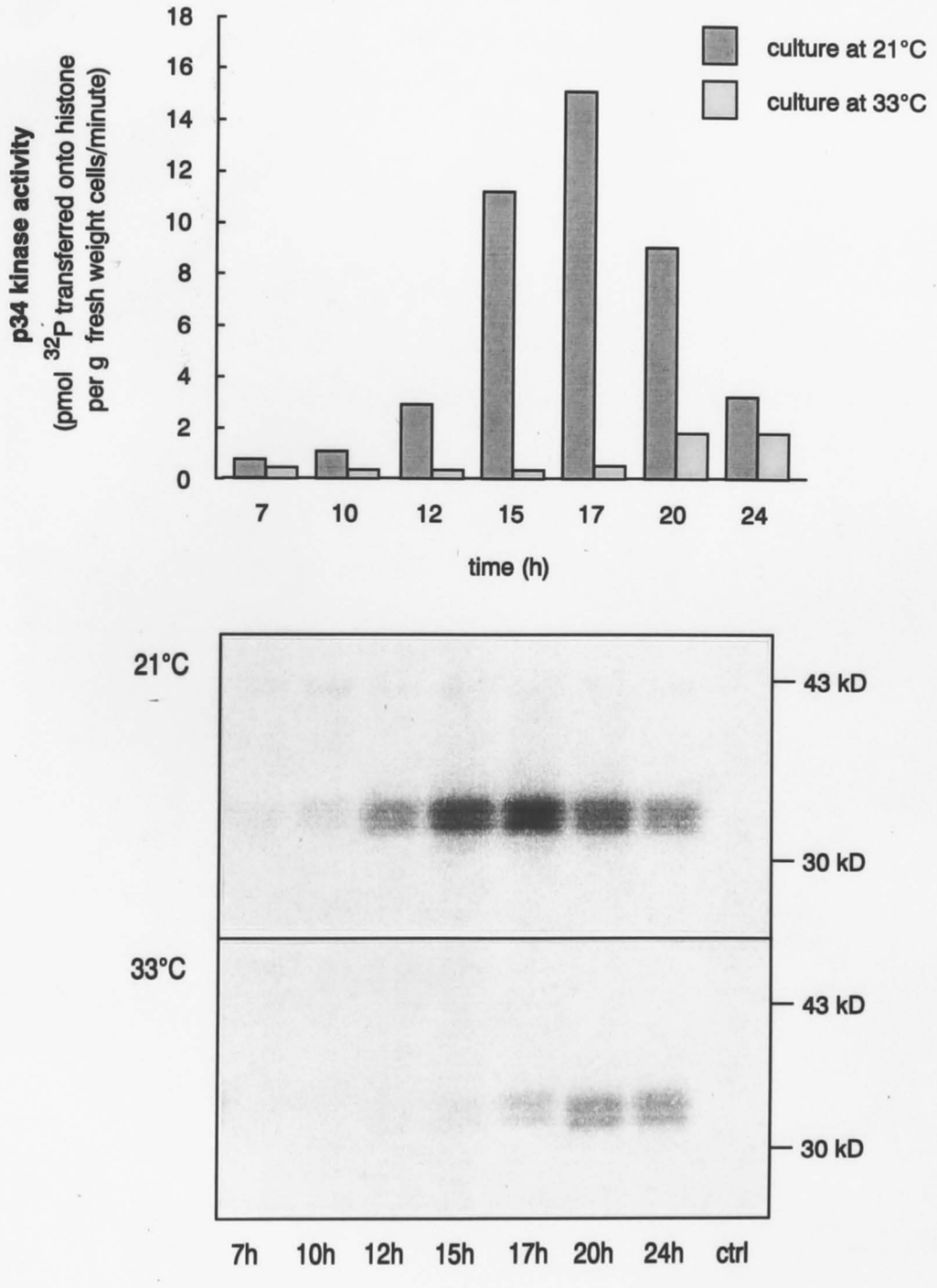
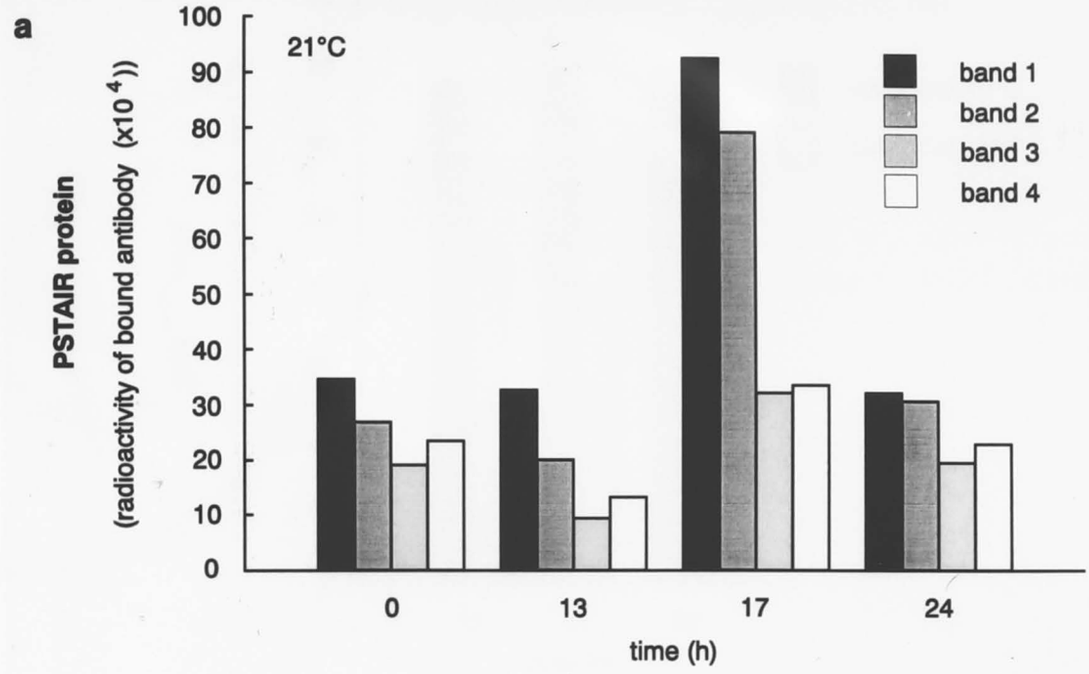
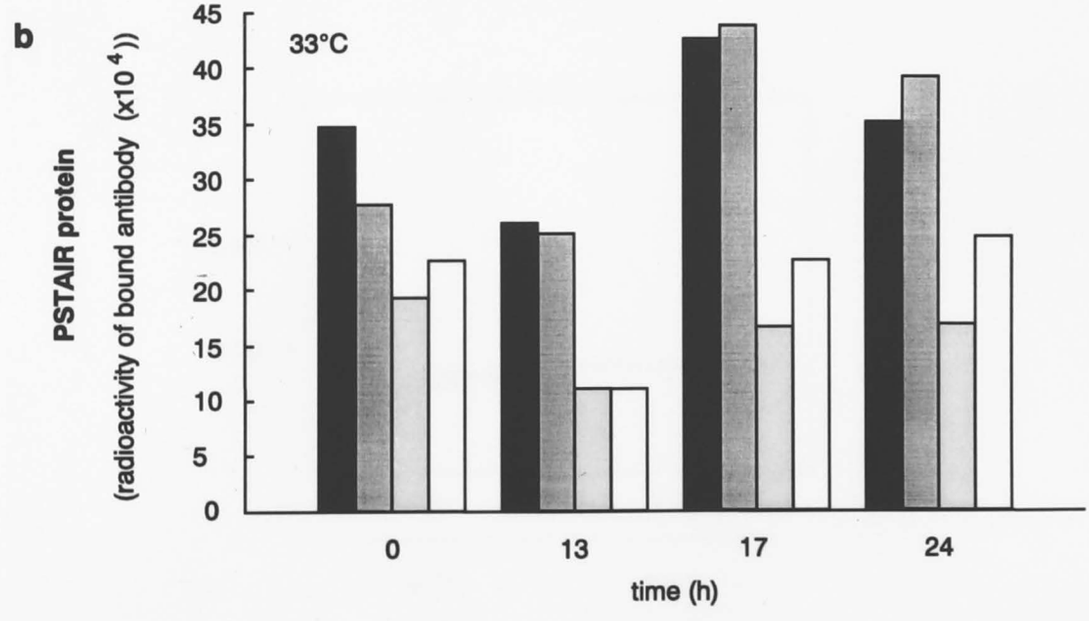
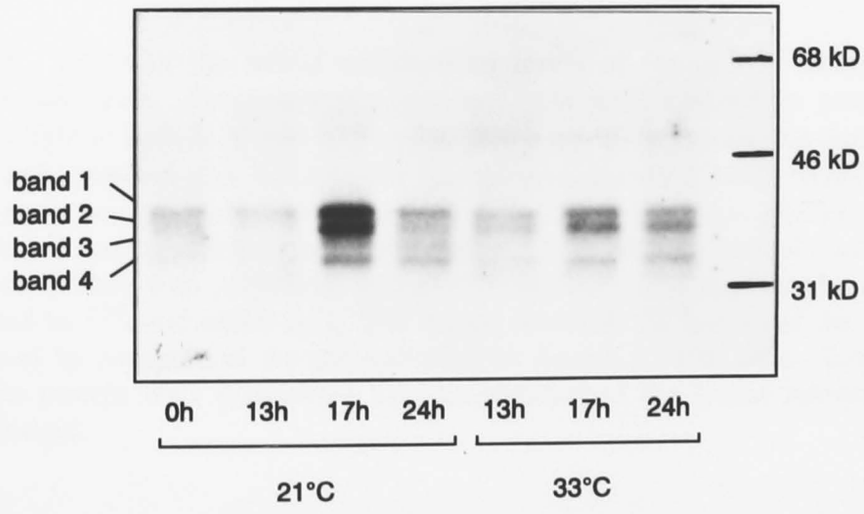

p34 kinase activity
(pmol ^{32}P transferred onto histone
per g fresh weight cells/minute)
18
16
14
12
10
8
6
4
2
0
culture at 21°C
culture at 33°C
7
10
12
15
17
20
24
time (h)
21°C
33°C
43 kD
30 kD
43 kD
30 kD
7h
10h
12h
15h
17h
20h
24h
ctrl

Fig. 3.3.9. Effect of the *cdM-1* mutation on the $p34^{cdc2}$-like protein levels during the cell cycle. Previously synchronised cells were cultured in parallel in continuous light at both 21°C and 33°C. Cell samples taken at various times during the cell cycle were extracted with RIPA buffer (see general method 2.2.15). Loadings of 50 µg total protein were separated on a 10-15% linear gradient SDS-acrylamide gel and transferred onto nitrocellulose. The Western blot was probed with anti-EGVPSTAIREISLLKE (PSTAIR) antibody and the bound antibody was detected by ^{125}I anti-rabbit IgG. The image, shown at the bottom of the figure, was obtained by exposure of the nitrocellulose onto Kodak X-omat film. Four bands were detected by the antibody, as shown in the bottom panel, and their levels were determined by quantification of the bound isotope on a PhosphorImager. Note that the ordinate scale is smaller in (b).


a
21°C
band 1
band 2
band 3
band 4
PSTAIR protein
(radioactivity of bound antibody (x10 4))
100
90
80
70
60
50
40
30
20
10
0
0
13
17
24
time (h)


b
33°C
PSTAIR protein
(radioactivity of bound antibody (x10 4))
45
40
35
30
25
20
15
10
5
0
0
13
17
24
time (h)


band 1
band 2
band 3
band 4
68 kD
46 kD
31 kD
0h
13h
17h
24h
13h
17h
24h
21°C
33°C

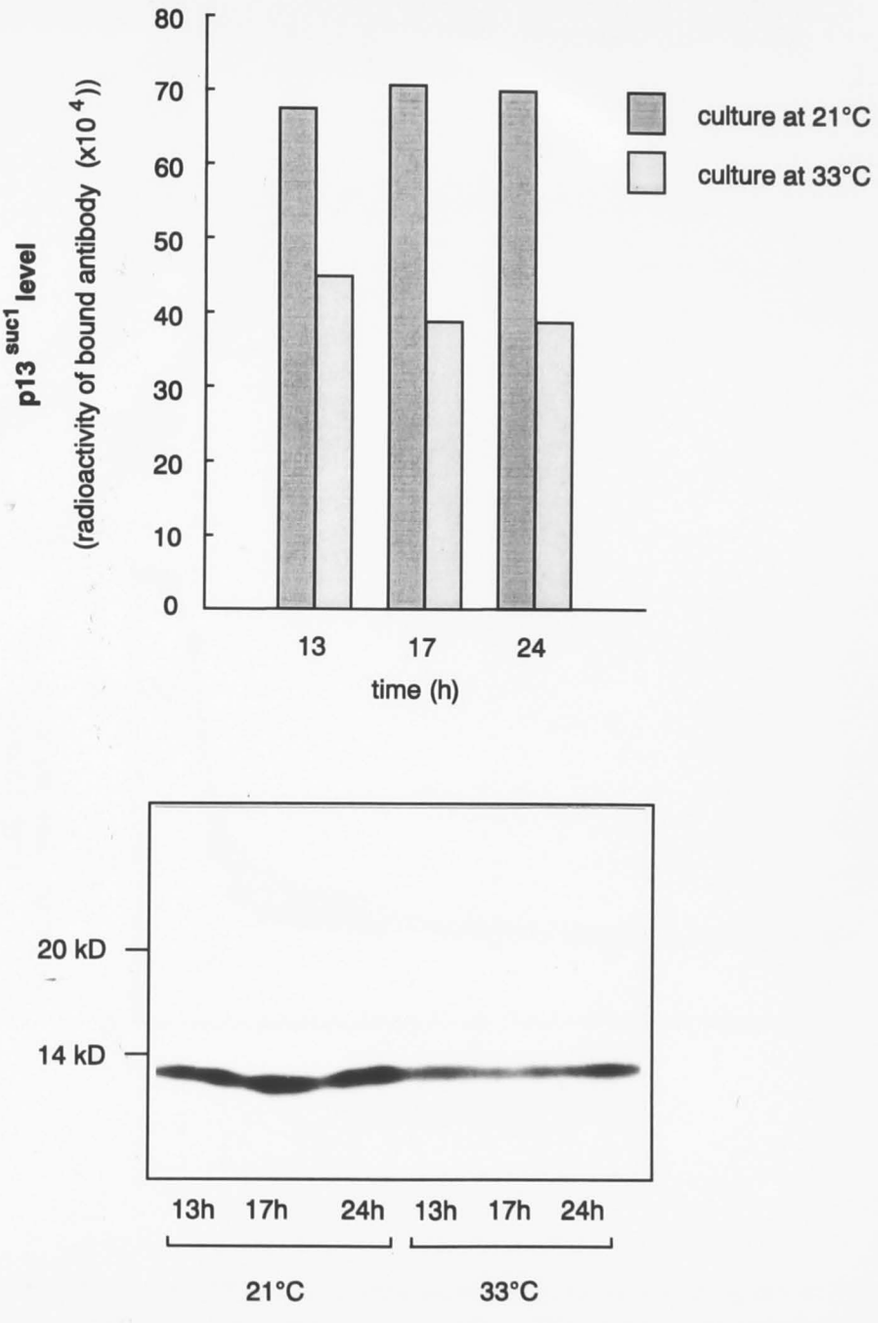


Fig. 3.3.10. Effect of the *cdM-1* mutation on levels of the p13^{suc1}-like protein during the cell cycle. Previously synchronised cells were cultured in parallel in continuous light at both 21°C and 33°C. Samples were taken at intervals during the cell cycle and extracted with RIPA buffer (see general method 2.2.15). Equal (50 µg) loadings of total protein were separated on a 10-25% linear gradient SDS-acrylamide gel and transferred onto nitrocellulose. The Western blot of transferred proteins was probed with polyclonal anti-p13^{suc1} antibody and the bound antibody was detected by ^{125}I anti-rabbit IgG. The image, shown at the bottom of the figure, was obtained by exposure of the nitrocellulose on Kodak X-omat film. Levels of p13^{suc1}-like protein were determined by quantification of the bound isotope on a PhosphorImager.

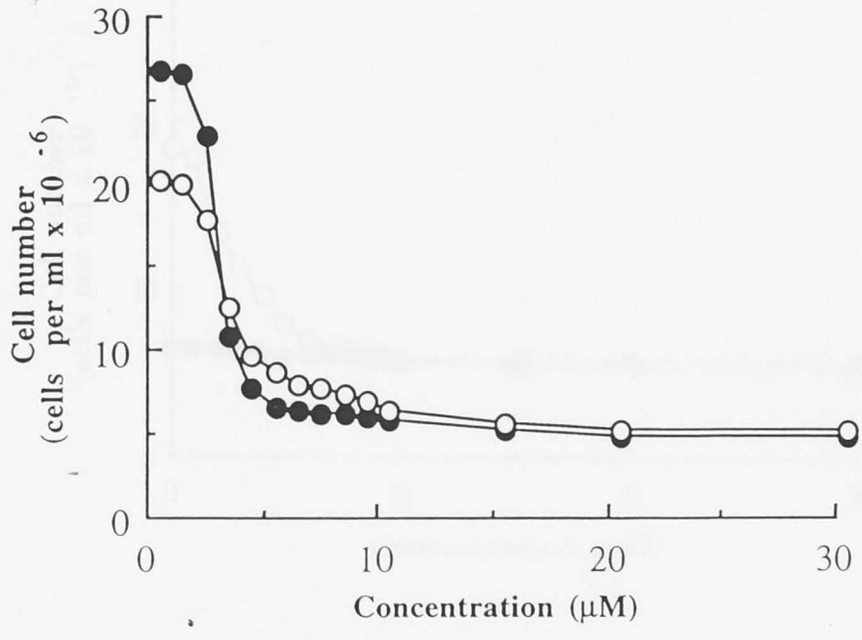


Fig. 3.3.11. Effects of different concentrations of oryzalin on progress to division in synchronous wild-type *Chlamydomonas* cells. Previously synchronised cells of wild-type *Chlamydomonas* (*CC-125*$^{+}$) were cultured in parallel from the beginning of the cell cycle at both 21°C (-○-) and 33°C (-●-) from an initial cell number density of 5 x 10^5 per ml. Cells from both cultures were treated in parallel with different final concentrations of oryzalin added at 7 h, just before cells attained commitment to divide. Each oryzalin treated subculture was fixed with 1% formaldehyde after one potential cell cycle duration (at 24 h). Cell number density in each oryzalin treated subculture was counted with a Coulter counter.

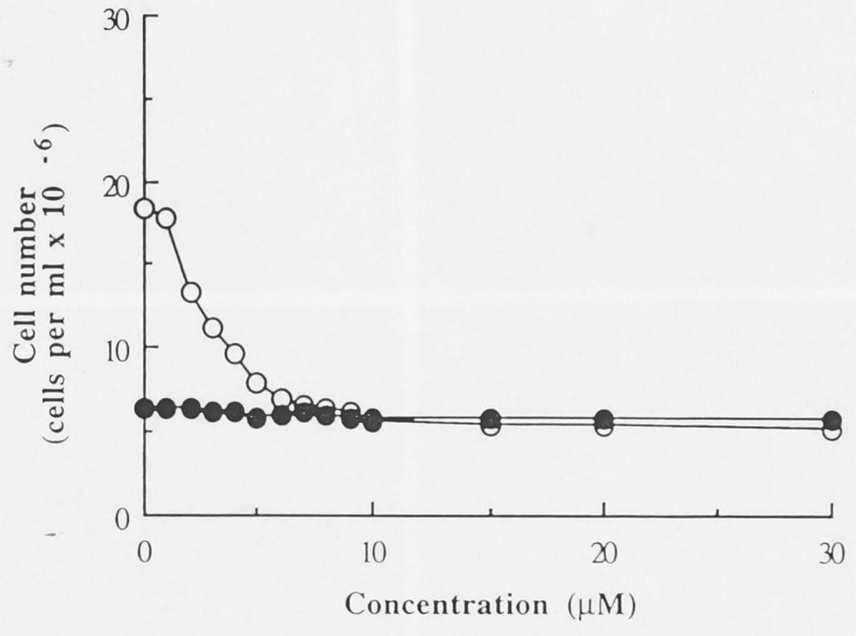


Fig. 3.3.12. Response of *cdM-1* in synchronous culture to different concentrations of oryzalin. Previously synchronised cells of *cdM-1* were cultured in parallel from 0 h of the cell cycle at both 21°C (-○-) and 33°C (-●-) from an initial cell number density of 5.5 x 10^5 per ml. Cells from both cultures were treated in parallel with different final concentrations of oryzalin added at 7 h just before cells attained commitment to divide. Each oryzalin treated subculture was fixed with 1% formaldehyde after one potential cell cycle duration (at 24 h). Cell number in each oryzalin treated subculture was counted with a Coulter counter.

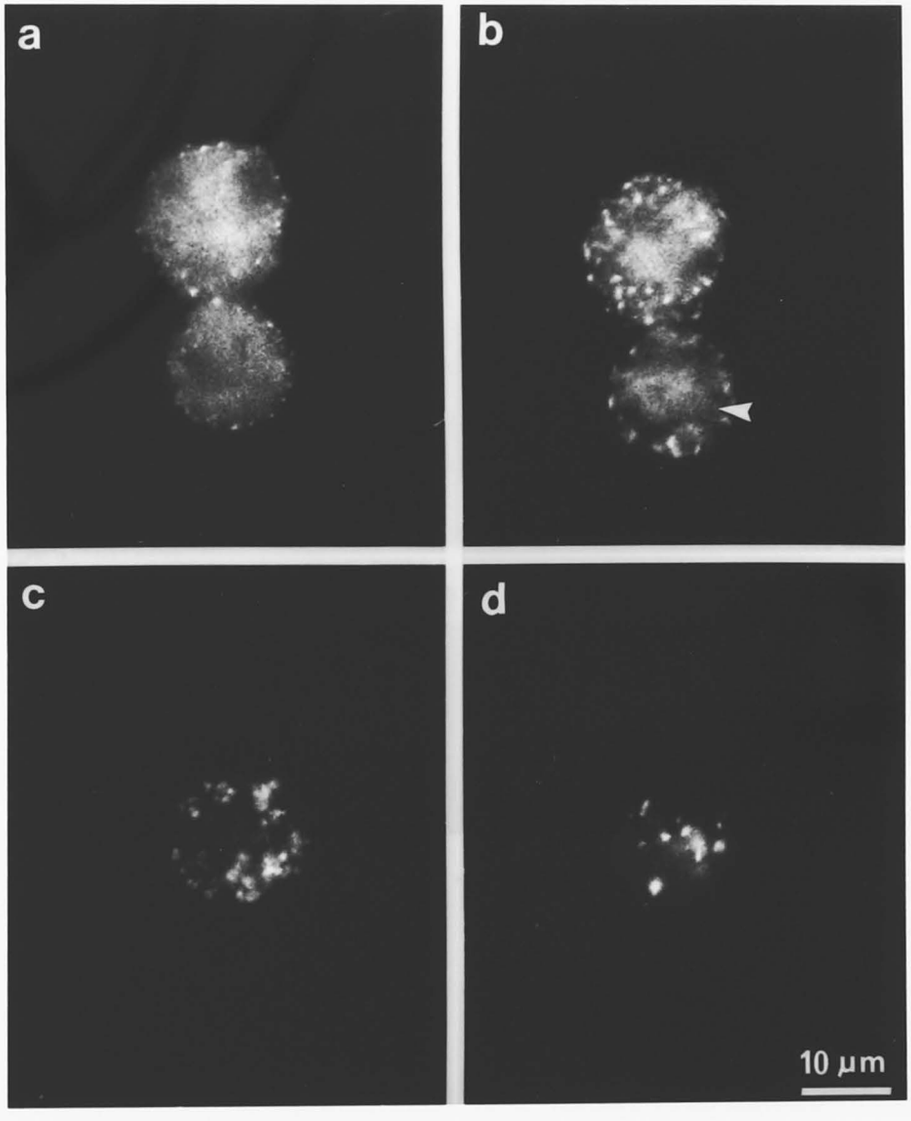


Fig. 3.3.13. Anti β-tubulin antibody staining of wild type and *cdM-1* cells arrested with 15 µM oryzalin at 21°C. Synchronous cells of wild type and *cdM-1* were cultured at 21°C. At 7 h oryzalin was added to a final concentration of 15 µM by addition of a 10 mM solution in DMSO. Cells were fixed at 24 h and stained with anti β-tubulin and DAPI. In (a), anti β-tubulin antibody staining of two wild type cells arrested with 15 µM oryzalin, showing the depolymerised β-tubulin in the arrested cells; (b), DAPI staining of the cells seen in (a), showing the single undivided nucleus in each cell; (c), anti β-tubulin antibody staining of oryzalin-arrested *cdM-1* cells at 21°C, showing the depolymerised β-tubulin in the arrested cell; (d), DAPI staining of the same cell in (c).

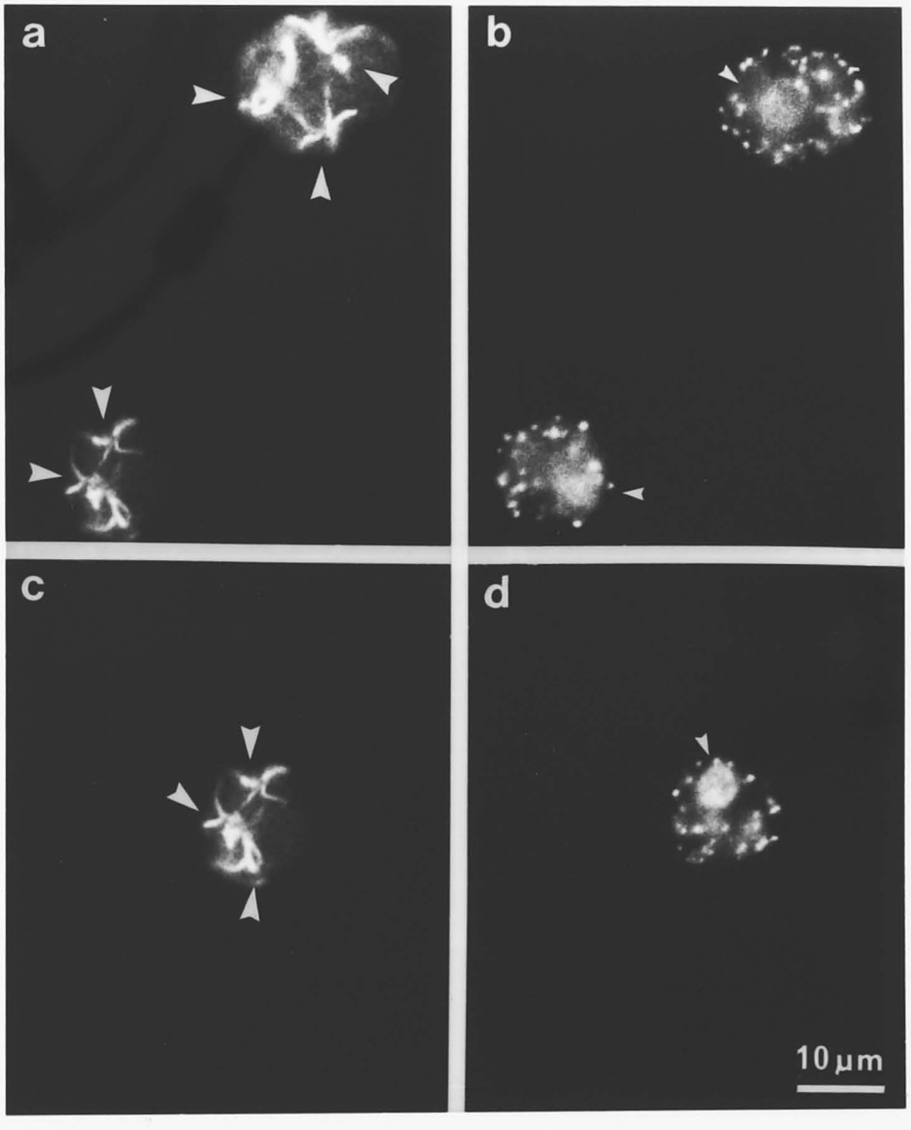


Fig. 3.3.14. Anti acetylated-tubulin antibody staining of oryzalin-arrested wild-type and *cdM-1* cells cultured at 21°C. Synchronous cells of wild type and *cdM-1* were cultured at 21°C. At 7 h oryzalin was added to a final concentration of 15 µM by addition of a 10 mM solution in DMSO. Cells were fixed at 24 h and stained with anti acetylated-tubulin and DAPI. In (a), anti acetylated-tubulin antibody staining of two wild type cells arrested by 15 µM oryzalin, showing the duplicated basal bodies each with four major flagellar roots seen as cruciate structures (arrows); (b), DAPI staining of the cells seen in (a), showing the single prophase nucleus in each cell (arrows); (c), anti acetylated-tubulin antibody staining of an oryzalin arrested *cdM-1* cell at 21°C, showing at least three basal bodies (a fourth is out of focus); (d), DAPI staining of the single cell seen in (c), showing the nucleus in this cell with four basal bodies.

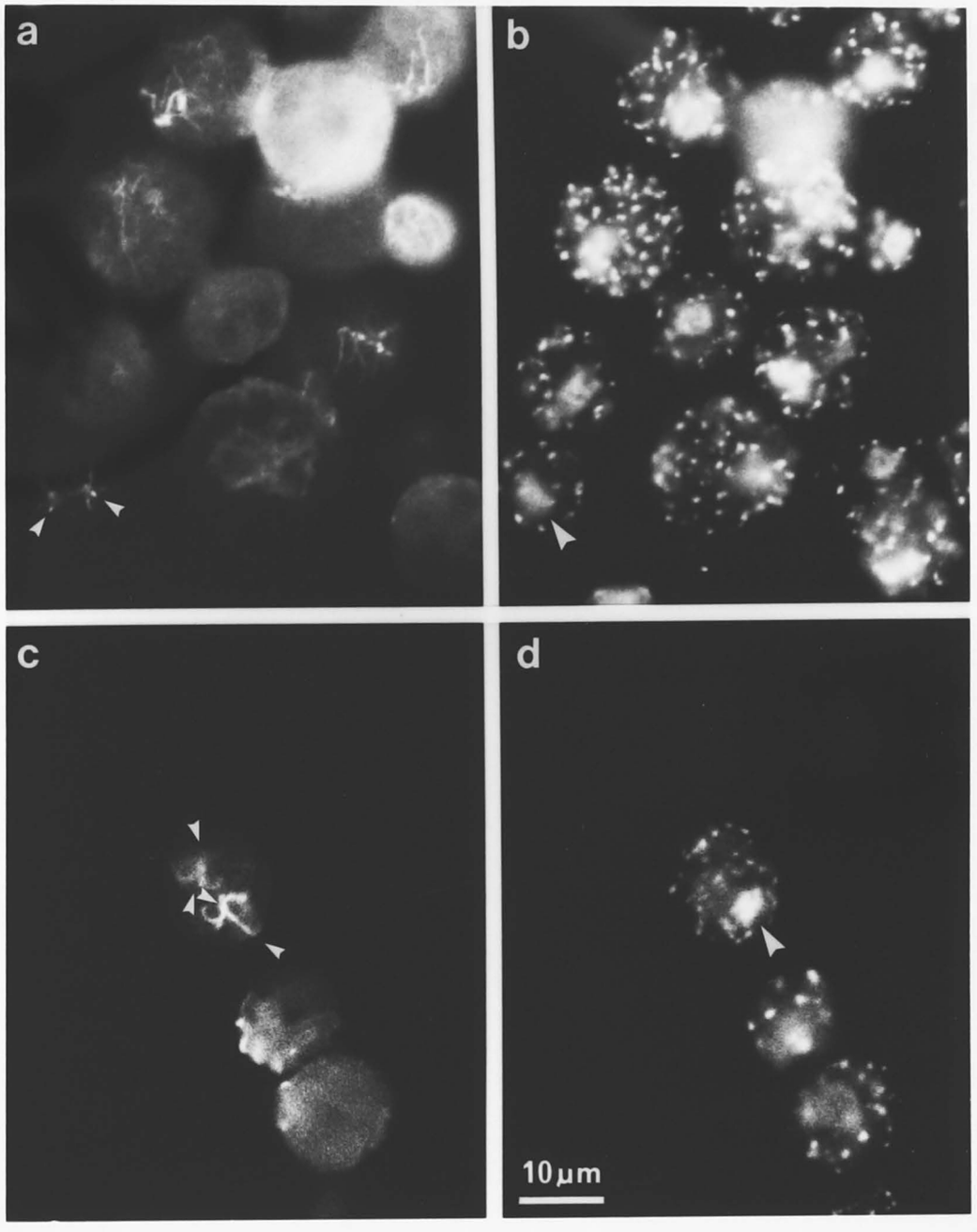


Fig.3.3.15. Anti acetylated-tubulin antibody staining of oryzalin-arrested wild type *Chlamydomonas* cells that were cultured at 21°C. (a), anti acetylated-tubulin antibody staining of oryzalin-arrested cells with multiple basal bodies, which are indicated by arrows, and single cells had 2-4 sets of basal body apparatus; (b), the cells in (a) stained with DAPI showing the position of prophase nucleus in each cell; (c), anti acetylated-tubulin antibody staining of oryzalin arrested cells, showing a cell with two sets of "metaphase bands" (running between the tips of the small arrow heads); (d), the cells in (c) stained with DAPI, showing the prophase nucleus with condensed DNA (large arrow head) in the cell that contains two "metaphase bands". Note that no metaphase band has formed without an adjacent nucleus.

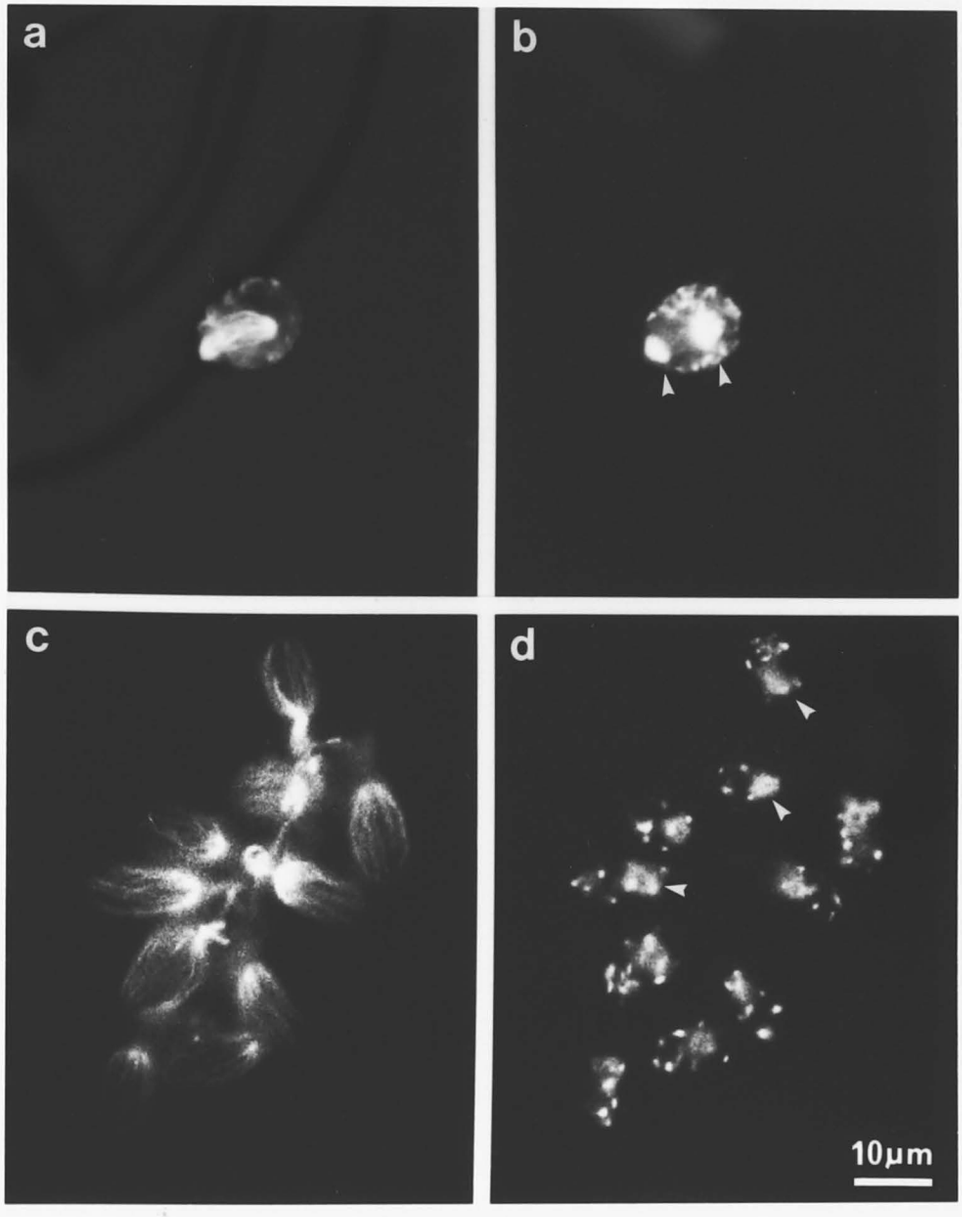


Fig. 3.3.16. Anti β-tubulin staining of wild type *Chlamydomonas* cells that were released from oryzalin inhibition. Synchronous cells were treated with 15 μM oryzalin from 7 h of the cell cycle and released from the drug at 16 h by washing twice in oryzalin-free TAPYPP medium and suspending in the same medium. (a), anti β-tubulin antibody staining of a cell that was fixed at 1 h after release from oryzalin, showing that an anaphase spindle has developed; (b), the cell in (a) stained with DAPI, showing the separated nuclear DNA in the anaphase cell; (c), anti β-tubulin antibody staining of the wild type cells that were released from oryzalin at 16 h and fixed at 24 h, showing newly divided daughter cells released from the recovered cells; (d), DAPI staining of the cells in (c), showing the position of the single nucleus in each cell.

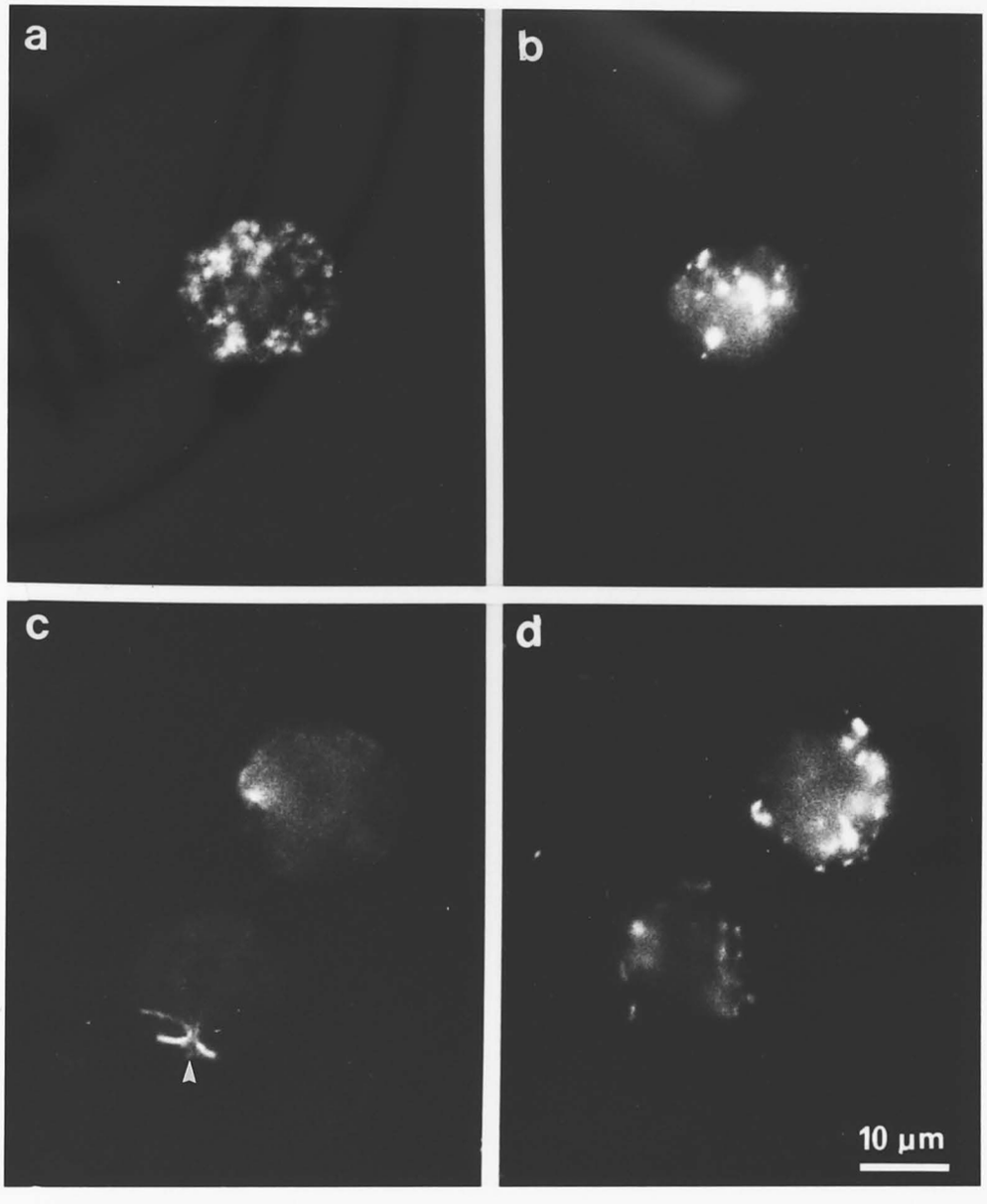


Fig. 3.3. 17. Anti β-tubulin and anti acetylated-tubulin antibody staining of oryzalin treated *cdM-1* cells that were arrested at 33°C. Synchronous cells at 33°C were treated with 15 µM oryzalin from 7 h then released from the drug at 16 h by two washes with oryzalin free TAPYPP medium, then resuspended in the same medium. Cells were fixed at 24 h and stained with antibodies. (a), anti β-tubulin antibody staining of a *cdM-1* cell after release from oryzalin treatment and culture at 33°C, showing the depolymerised cortical microtubules but persistence of arrest at the restrictive temperature; (b), DAPI staining of the cell in (a) showing the poor staining characteristic of continuing arrest to 24 h; (c), two *cdM-1* cells stained with anti acetylated-tubulin antibody, showing that after release from the oryzalin treatment and continued incubation at 33°C cells were still arrested in G2 phase with an unduplicated basal body (arrow); (d), DAPI staining of the cells in (c), the nucleus again stained poorly with DAPI characteristic of continuing arrest to 24 h.

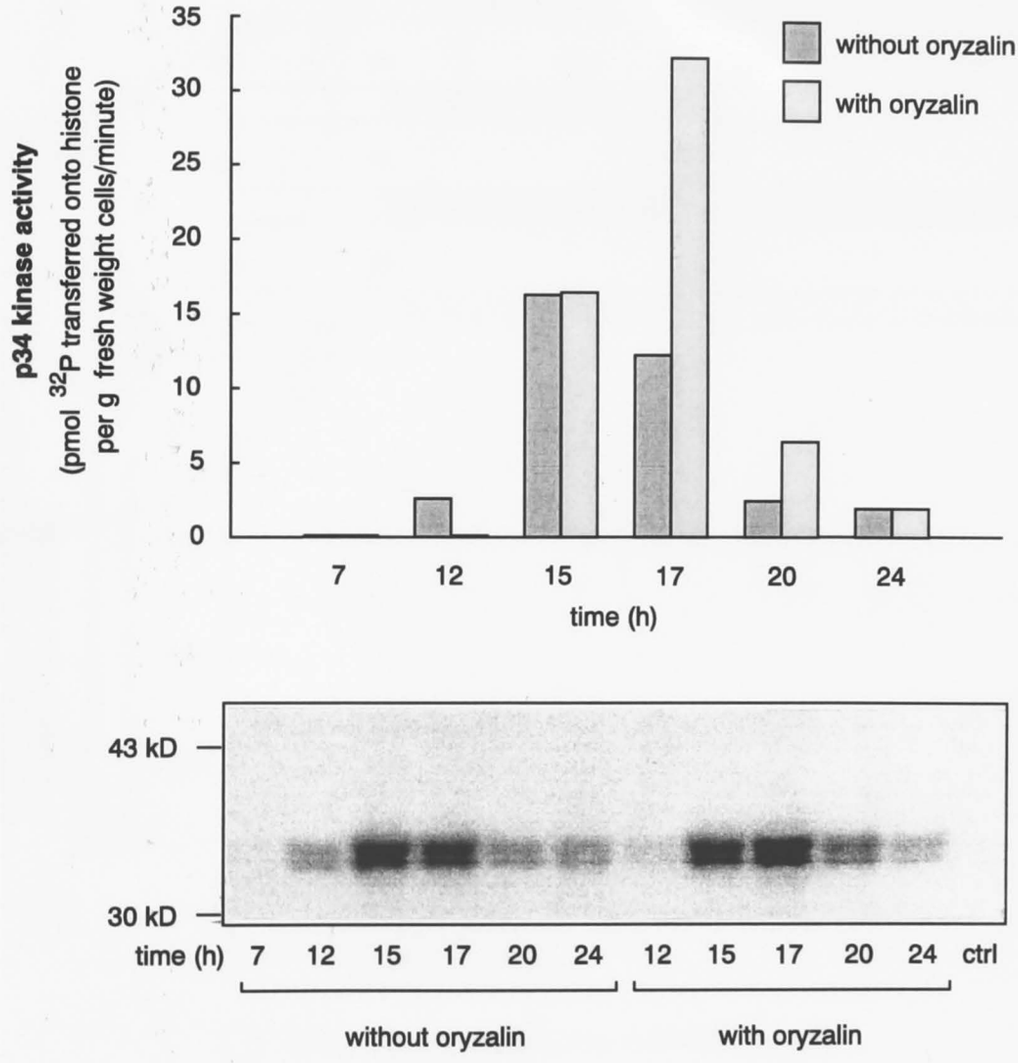


Fig. 3.3.18. Effect of 15 µM oryzalin on p34^{cdc2}-like protein kinase activity during the cell cycle of synchronous wild-type *Chlamydomonas* (*CC-125*$^{+}$). Previously synchronised wild-type cells were cultured in continuous light at 21°C and sampled from the beginning of growth in newly formed daughter cells. At 7 h oryzalin was added to a final concentration of 15 µM by addition of a 10 mM solution in DMSO. The oryzalin-treated and untreated control cultures were sampled in parallel for one cell cycle duration. Samples of 0.01 g of fresh weight of cells ground in liquid nitrogen were extracted with NDE buffer (see General Method 2.2.19). The p34^{cdc2}-like protein kinase was purified from each sample by binding to 20 µl of p13^{suc1} beads, followed by two washes with detergent bufer and one wash without detergent then eluting with 50 µl of 0.5 mg/ml of free p13^{suc1} solution. The activity of purified p34^{cdc2}-like protein kinase was measured using histone H1, as a substrate at 30°C for 5 minutes. The activity is illustrated as the amount of ^{32}P transferred onto histone which was measured by placing 20 µl of reaction mixture on P81 phosphocellulose paper, washing with 75 mM phosphoric acid and then counting in a scintillation counter. The radioactivity of labelled phospho-histone H1, shown in the bottom panel of the figure, was obtained by separation of 30 µl of reaction mixture on a 12% acrylamide gel and then exposure in a PhosphoImager.

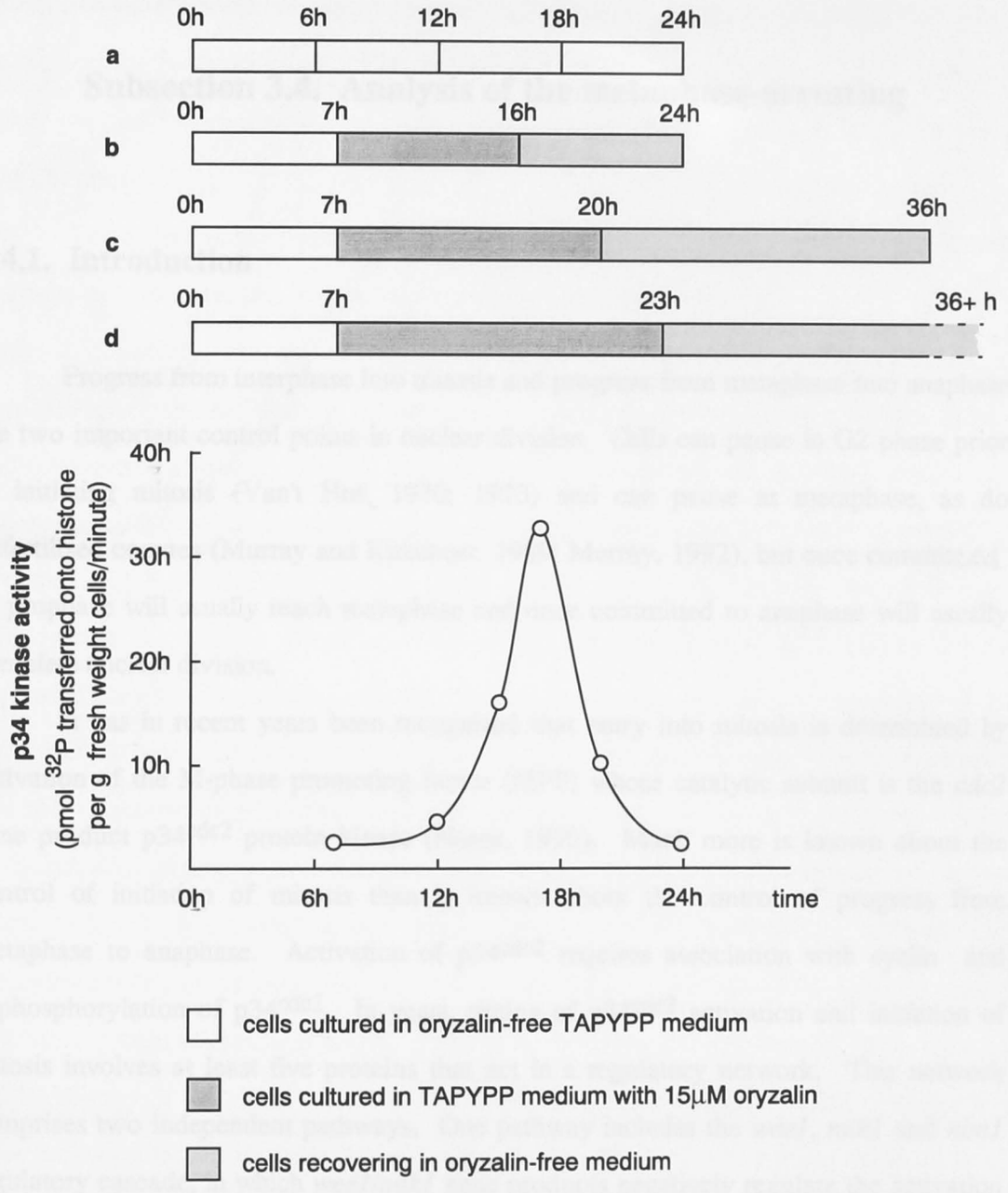


Fig. 3.3.19. The relationship of ability to recover from oryzalin treatment and the residual $p34^{cdc2}$-like protein kinase activity present in the cells at the time transfer to oryzalin-free medium. Activity in cells remaining in oryzalin is shown in the in the centre panel. Synchronous wild type cells were cultured at 21°C and at 7 h oryzalin was added to a final concentration of 15 μM by addition of a 10 mM solution in DMSO. Cells were released from oryzalin at 16 h, 20 h and 23 h by two washes in oryzalin-free TAPYPP medium and then resuspension in the same medium. The time required for recovery from the drug to completion of cell division and release of daughter cells was observed. (a), control cells without oryzalin treatment, released all daughter cells at 24 h; (b), cells freed from inhibition at 16 h, completed cell division and released daughter cells at 24 h; (c), cells that were freed from oryzalin at 20 h completed cell division at 32 h and released daughter cells at 36 h; (d), cells that were freed from oryzalin at 23 h had not initiated any mitosis by 36 h.

Subsection 3.4. Analysis of the metaphase-arresting mutant *met-1*

3.4.1. Introduction

Progress from interphase into mitosis and progress from metaphase into anaphase are two important control points in nuclear division. Cells can pause in G2 phase prior to initiating mitosis (Van't Hof, 1970; 1973) and can pause at metaphase, as do unfertilised oocytes (Murray and Kirschner, 1989; Murray, 1992), but once committed to prophase will usually reach metaphase and once committed to anaphase will usually complete nuclear division.

It has in recent years been recognised that entry into mitosis is determined by activation of the M-phase promoting factor (MPF) whose catalytic subunit is the *cdc2* gene product $p34^{cdc2}$ protein kinase (Nurse, 1990). Much more is known about the control of initiation of mitosis than is known about the control of progress from metaphase to anaphase. Activation of $p34^{cdc2}$ requires association with cyclin and dephosphorylation of $p34^{cdc2}$. In yeast, timing of $p34^{cdc2}$ activation and initiation of mitosis involves at least five proteins that act in a regulatory network. This network comprises two independent pathways. One pathway includes the *wee1*, *mik1* and *nim1* regulatory cascade, in which *wee1/mik1* gene products negatively regulate the activation of $p34^{cdc2}$ protein kinase and the *nim1* gene product acts as an inhibitor of wee1/mik1 and which is therefore a positive regulator of the $p34^{cdc2}$ at the G2/M transition (Russell and Nurse, 1987a, 1987b). The other pathway includes the *cdc25* gene product which is required for dephosphorylation and activation of the $p34^{cdc2}$ kinase (Russell and Nurse, 1986). In *Aspergillus*, it has been found that initiation of mitosis requires not only $p34^{cdc2}$ protein kinase but also an additional protein kinase encoded by the *nimA* gene (Osmani et al., 1987; Morris, 1990). Recent investigations have revealed that activation of the NIMA kinase is independent of the activation of $p34^{cdc2}$ protein kinase, and activation of $p34^{cdc2}$ is not dependent on the NIMA kinase activity (Osmani et al., 1991).

These two independent protein kinases act in parallel to regulate the initiation of mitosis in *Aspergillus* (Osmani et al., 1991, Morris and Enos, 1992; Doonan, 1992). Lack of either the $p34^{cdc2}$ kinase activity or the NIMA kinase activity results in lethal arrests in late G2 phase.

Fewer mutations have been found to cause arrest in mid nuclear division and, perhaps in consequence, less is known about the mechanisms that control exit from mitosis. In many cells the metaphase to anaphase transition is tightly regulated by feedback controls that prevent the onset of anaphase until the mitotic spindle has been fully assembled (Murray, 1992). The molecules that mediate the feedback control are not known. Studies in *Xenopus* suggest that cyclin degradation is a key step governing progress beyond metaphase to exit from mitosis and progress into next cell cycle (Glotzer et al., 1991). Modified cyclins that can activate the MPF but cannot be degraded result in persistently active MPF and arrest at metaphase. A region called the "destruction box" motif on the N-terminal half of cyclin has been found to be necessary for the degradation of cyclins and can be recognised by some component of the ubiquitin-conjugating system (Glotzer et al., 1991). Introduction of mutant cyclin that lacks the 90 N-terminal amino acids prevents MPF inactivation, chromosome decondensation, cytokinesis and spindle disassembly. This indicates that these late mitotic events are dependent on the cyclin B degradation and subsequent inactivation of MPF (Murray et al., 1989). It is not clear what triggers the ubiquitination of cyclin and how the degradation of cyclin is regulated to occur after, but not before, metaphase. A recent investigation in budding yeast revealed that anaphase can occur in the presence of high levels of CDC28/CLB2 mitotic kinase activity in a *cdc15* mutant, and also that either overexpression of wild-type cyclin or expression of nondegradable cyclin causes cells to arrest in telophase (Surana et al., 1993), rather than at the earlier metaphase arrest point identified in *Xenopus* oocytes (Murray et al., 1989). It has also been recently observed that addition of a nondegradable form of mutant cyclin B to frog egg extracts, which prevents MPF inactivation as observed by Murray et al (1989), does not block the early stages of sister chromatid separation, indicating that MPF inactivation is not required for initiation of anaphase. However, adding an N-terminal fragment of cyclin as

a specific competitor for cyclin degradation, or adding methylated ubiquitin that inhibits ubiquitin-mediated proteolysis, does delay MPF inactivation and sister chromatid separation (Holloway, et al. 1993). This finding suggests that anaphase is initiated by proteolysis of cyclin rather than by the inactivation of MPF. It is still obscure what mechanism controls the timing of cyclin proteolysis. The metaphase-arresting mutant *met-1* may provide a useful experimental system for investigation of the metaphase-anaphase transition.

3.4.2. Results

3.4.2.1. Commitment point of the *met-1* mutant

Cells of *met-1* in synchronous culture at 21°C attained first commitment to divide at a mean time of 11 h, and completed their cycle at 20 h (Fig.3.4.1.A). At the restrictive temperature, cells became blocked at mitotic metaphase and no daughter cells were produced (Fig.3.4.1.B). The mean time of first commitment in the cells that were cultured at 33°C was at 11 h. At this time, in both 21°C and 33°C cultures, 50% cells of the synchronous population were able to divide on transfer to zero growth as indicated by an increase in the total cell number of 1.5 fold relative to the initial cell number (Fig.3.4.1.B).

3.4.2.2. The execution and catastrophe points of the *met-1* mutant

Data from temperature shifting experiments showed that when synchronous cells of the *met-1* mutant were incubated at 33°C from the beginning of the cell cycle at time 0 h, and were then transferred back to 21°C at the times plotted (Fig.3.4.2), the cells that were shifted before 13 h retained full capacity to complete the current cell cycle and produce daughter cells on transfer to 21°C. Thereafter cells in the synchronous

population lost the capacity to complete the cell cycle and had begun to traverse the "catastrophe point", which in most cells occurred at 15 h and was about 4 h after the commitment point (Fig.3.4.2). When the cells were transferred from 21°C to 33°C at the times plotted (Fig. 3.4.2), the cells in the synchronous culture that were shifted after 15 h began to attain the ability to complete the current cell cycle in spite of a potentially restrictive temperature and fully attained this capacity by 18 h. The mean time at which cells in the synchronous population acquired the capacity to complete the cell cycle under restrictive temperature was at 17 h. The execution point for *met-1* function under these conditions was 6 h after the commitment point (Fig. 3.4.2.). The time difference between the "catastrophe point" and the "execution point" in the synchronous population of the *met-1* mutant is 2 hours, during which cells normally complete two rounds of mitosis but in which at restrictive temperature the mutated *met-1* gene product would make its faulty contribution to the cell cycle and cause the metaphase block.

3.4.2.3. The effect of the *met-1* mutation on the $p34^{cdc2}$-like protein kinase

A key mitotic protein is the cell cycle control protein $p34^{cdc2}$ (reviewed by Nurse, 1990 and the introduction) and since $p34^{cdc2}$-like protein has been detected in *Chlamydomonas* and shown to undergo change in level and phosphorylation in the normal cell cycle (John et al., 1989), it is interesting to check the effect of the *met-1* mutation on this key cell cycle regulating protein. *met-1* cells from the same parent culture were grown at both permissive and restrictive temperatures so that a comparison could be made between the cells that could undergo normal cell division and the cells that blocked at mitotic metaphase at the restrictive temperature. Samples were taken at the times as indicated (Fig.3.4.3 , 3.4.4 and 3.4.5) and monitored for mitotic index, $p34^{cdc2}$ kinase activity and level of $p34^{cdc2}$ protein during the cell cycle.

DAPI staining provided a useful tool for rapid detection of condensed chromosomes that are indicative of mitosis. At permissive temperature a peak of about 50% mitotic cells was observed at about 13 h of the cell cycle and then declined as cells proceeded to cytokinesis and completion of cell division (Fig.3.4.3). In the culture at 33

°C mitotic cells started to appear with similar timing at 12 h, and increased to more than 70% at 13 h, reaching about 80-90% by 24 h, indicating that essentially all of the cells became arrested at the restrictive temperature (Fig. 3.4.3.).

At the permissive temperature, the $p34^{cdc2}$-like histone H1 kinase activity recovered by affinity purification on p13 beads peaked coincidentally with the mitotic index, being low from 0-10 h, reaching a high level at 13 h of the cell cycle and then declining to the low level by 20 h. When cells were incubated at restrictive temperature, the histone H1 kinase activity was again low from 0-10 h, rising to a high level at 13 h, which was a little higher than at the mitotic peak in cells at 21°C, and in marked contrast continued to increase reaching the very high level of about twice as high as the maximum in unarrested cells at 21°C (Fig.3.4.4). The elevated activity correlated with failure to progress through anaphase.

The level of $p34^{cdc2}$-like protein in *met-1* cells was assayed using antibody against PSTAIR sequence of $p34^{cdc2}$. As observed in wild type cell extracts, four distinctive bands of $p34^{cdc2}$-like proteins were detected in the *met-1* cell extracts from both permissive and restrictive temperatures. The level of the total $p34^{cdc2}$-like proteins during the cell cycle at 21°C fluctuated in a similar way to the $p34^{cdc2}$ kinase activity in the same culture, which increased about 3 fold by 13h, and then declined to the initial G1 phase level (Fig. 3.4.5.). At 33°C, the level of the total $p34^{cdc2}$-like proteins increased as the cells entered and became arrested in mitosis at 13 h reaching twice control levels (21°C levels) at this time and although falling by 30% between 13 h and 20 h (Fig.3.4.5) remaining twice as high as at 21°C. Considering each band of the $p34^{cdc2}$-like proteins, it seems that the level of the band 3 protein both in cells at 21°C and at 33°C is relatively constant through the cell cycle. However, the band 1 protein increased at G1/S and band 2 protein increased at mitosis, both declining when S phase and mitotic activity were completed. The decline is part of normal cell cycle progress since it was prevented in the arrested cells. It remains to be determined whether all of the PSTAIR bands are different phosphorylation states of a single $p34^{cdc2}$ protein, or whether other members of the cdc2 protein family are present in *Chlamydomonas*.

3.4.2.4. Effect of the *met-1* mutation on the level of $p13^{suc1}$

Investigations in the yeast cell cycle have revealed that presence of $p13^{suc1}$ is necessary for inactivation of $p34^{cdc2}$ at anaphase and for cells to exit from mitosis (Moreno et al., 1989). Raised levels of $p13^{suc1}$ can suppress some mutations in the *cdc2* gene of *S. pombe* (Hayles et al., 1986; Nurse, 1990). Since *met-1* arrested at metaphase with high levels of $p34^{cdc2}$ kinase activity, it was of interest to ascertain whether the $p13^{suc-1}$ like protein in the arrested cells was altered in comparison with that of cells dividing at 21°C. To analyse $p13^{suc-1}$ levels, cell extracts were made from samples that had been taken at the same times of the cell cycle as that for observation of the mitotic index and analysis of $p34^{cdc2}$-like protein (Fig. 3.4.6.). Using polyclonal antibody against the *S. pombe* $p13^{suc1}$, a single band of $p13^{suc1}$-like protein with a mobility consistent with 13 kDa molecular size was detected on the Western blot of *met-1* cell extracts from cells cultured at both 21°C and 33°C. The level of the $p13^{suc1}$-like protein was measured by quantification on a PhosphorImager. Unlike the $p34^{cdc2}$-like proteins, levels of the $p13^{suc1}$-like protein in the arrested *met-1* cells were similar to those in the cells at 21°C and the levels in the cells at both conditions changed little throughout the cell cycle, indicating that the level of $p13^{suc1}$-like protein was not affected by the *met-1* mutation.

3.4.2.5. Effect of the *met-1* mutation on the level of $p56^{cdc13}$-like cyclin protein

Cyclins are a class of regulatory proteins that can bind to the $p34^{cdc2}$ protein kinase. Cyclins have a higher affinity for $p34^{cdc2}$ than $p13^{suc1}$ has, and cyclins are believed to play a key role in directing the catalytic subunit $p34^{cdc2}$ to specific substrates in particular cell cycle phases. The mitotic form of $p34^{cdc2}$ kinase is associated with cyclin-B. When cells enter the final stages of mitosis this kinase must be inactivated and the degradation of the cyclin is required for this inactivation of $p34^{cdc2}$ kinase. After the detection of persisting high $p34^{cdc2}$ kinase activity in the arrested *met-1* cells, it was

therefore important to test whether the cells were able to degrade their mitotic cyclin at the restrictive temperature. Using an antibody against the cyclin B ($p56^{cdc13}$) from *S. pombe*, a 56 kDa protein was detected in the electrophoretically resolved protein samples from both the mitotic cells at 21°C and the metaphase-arrested cells at 33°C. When a preimmune serum was tested against the same samples on the Western blot the 56 kDa band was not detected (Fig. 3.4.7). To establish the relationship between this $p56^{cdc13}$-like protein and the $p34^{cdc2}$ inactivation synchronous cells from the inoculum were incubated at 21°C and 33°C from 0 h of the cell cycle and sampled at the same times as for $p13^{suc1}$ estimation and $p34^{cdc2}$ kinase assay. At the permissive temperature, the level of $p56^{cdc13}$-like protein (cyclin B) peaked at 13 h, and the time of peak mitotic activity then sharply declined (Fig. 3.4.7). The level of cyclin B like protein in the cells that had progressed normally though G1, S and G2 phase but arrested in mitosis at the restrictive temperature also increased at 13 h but the high level of cyclin B persisted until the end of the sampling period (Fig. 3.4.7) The fluctuation of the cyclin B like protein in cycling cells was perfectly coincident with the oscillation of the $p34^{cdc2}$ kinase activity and mitotic activity and suggests that in plants as in other eukaryotes degradation of cyclin B like protein is essential for inactivation of the $p34^{cdc2}$ kinase and thus for exit from mitosis.

3.4.2.6. Induction of mitosis in higher plant cells by MPF from the *met-1* mutant

The failure to inactivate the $p34^{cdc2}$ kinase in the *met-1* cells might be caused by inability to degrade the cyclin B like protein under restrictive conditions. This kind of situation has been documented in some other organisms. For example, metaphase arrest in *Xenopus* can be obtained by microinjection of mRNA for a cyclin B lacking the "destruction box" into *Xenopus* egg extracts (Murray, 1987; Murray and Kirschner, 1989). Expression of a gene for nondegradable cyclin B causing arrest at a late stage of mitosis has also been reported in *S. cerevisiae* (Surana et al., 1993). Alternatively if the $p34^{cdc2}$-like kinase activity is of altered specificity in the mutant and able to phosphorylate H1 histone but not other essential substrates, this might account for mitotic

arrest. If the failure of initiating anaphase and completing mitosis in the *met-1* mutant is due to the production of a nondegradable mitotic cyclin or due to an altered specificity of the kinase, then MPF from the arrested *met-1* cells would not be able to function normally in other experimental systems. Active $p34^{cdc2}$ kinase purified by affinity binding to $p13^{suc1}$ can be readily derived from synchronous *met-1* cells that have been uniformly arrested at mitotic metaphase with high $p34^{cdc2}$ kinase activity. A sample taken at 17 h from the metaphase arresting synchronous culture was used to make a cell extract for affinity purification of MPF. After binding to $p13^{suc1}$ coupled sepharose beads and being eluted with free $p13^{suc1}$ protein, the concentrated MPF purified from arrested *met-1* cells achieved an extremely high activity which was more than 20 fold that from synchronous mitotic cells of the wild type *Chlamydomonas*. When the purified active MPF was microinjected (under a confocal microscope) into the early prophase stamen hair cells of the higher plant *Tradescantia virginiana*, which would normally stay in the early prophase for several hours without proceeding in mitosis, the injected cells were rapidly induced to undergo unusually extensive early chromosome condensation, and nuclear envelope breakdown began locally near to the site of the injection within 8-12 min and was complete only 33 min after the injection. This was followed by normal formation of the mitotic spindle, chromosome separation and then phragmoplast formation (Fig. 3.4.8.). This successful cell division induced by the MPF purified from the arrested *met-1* cells not only revealed full function of the *met-1* MPF in initiation of mitosis but also indicated that the mitotic cyclin in the MPF complex of the arrested *met-1* cells was degradable. In the *met-1* mutant cells therefore failure in initiating anaphase was not caused by an inherently nondegradable cyclin B like protein, rather it might be due to an earlier block in a signal for the cyclin destruction or due to a failure of the machinery of MPF inactivation in the mutant cells as discussed more fully in the final discussion.

3.4.2.7. Suppression of the *met-1* gene function

Isolation of suppressor mutations is one way to identify other proteins that interact with the mitotic protein that has been identified by the *met-1* mutation and

eventually to gain more information about the network in which it acts. This strategy has been used in the past for example, successfully to identified *suc1*$^+$ as a gene encoding a suppressor of some *cdc2*$^+$ mutations in *S. pombe* (Hindley et al., 1987).

To isolate suppressors from *met-1*, cells were synchronously cultured at 21°C then treated with 180 mM EMS for 1 hour during second S phase to cause additional mutation and transferred into fresh medium to recover overnight,then plated at restrictive temperature for suppressor screening. Cells with the original phenotype would be unable to form colonies. Two colonies were obtained which were able to undergo cell division at 33°C. When the two cell lines derived from these colonies were backcrossed with wild type cell lines, a 2:2 or 3:1 ratio of wild type : *met-1* phenotypes were observed. This result indicated that back mutation of the original *met-1* mutation was not responsible for suppression. Rather an additional suppressing mutation was present. Results from back crosses also revealed that when the second mutation was present in the absence of the *met-1* mutation, the cells had no phenotype that affected division and appeared to be similar to the wild type cells. Although lack of their own phenotype raised complication for further analysis of the suppressors, the identification of the suppressors itself has suggested that the gene product identified by the *met-1* mutation does act together with some other proteins for the essential function at the metaphase-anaphase transition. A more extensive screen might well yield suppressors with sufficiently crucial roles in mitosis in forms sufficiently altered by mutation to cause an arrest of division, but time did not allow this to be pursued.

3.4.2.8. Chromosome mapping of the *met-1* mutation

Mapping of the *met-1* mutant gene was carried out to establish its spatial relationship with other genes and to investigate the possibility of using a linked marker as an aid to cloning the gene as would be possible if the marker corresponded with a cloned gene or RFLP fragment that might be used to probe a library. Nineteen chromosome linkage groups have been reported in *Chlamydomonas reinhardtii* (see Harris, 1989; and the nuclear gene map presented in Fig. 3.4.9). More recently Dutcher's group have

amended this number to seventeen by reuniting XII and XIII as a single linkage group and XVI and XVII as another single linkage group (Dutcher, et. al, 1991).

To map the *met-1* mutation to a particular linkage group, the mutant cells were crossed with mapping strains that carry known markers for each of the 17 linkage groups. For testing of the linkage between *met-1* and each marker, tetrads from 50-100 zygospores were analysed. Data derived from tetrad analysis from crosses between *met-1* and loci for each linkage group are presented in Table 3.4.1.

Table 3.4.1 shows that, except that the mapping strain carrying markers for the linkage group V refused to mate with the *met-1* mutant, 50-100 zygospores were scored for each cross between *met-1* and each of the other markers. Linkage was determined according to the frequencies of parental ditype (PD), nonparental ditype (NPD), tetratype (T) and the mapping distance or recombination frequency (RF) was calculated by the equation:

RF (%) = (NPD+0.5T)/(PD+NPD+T)x100 (Suzuki et al., 1981; Harris, 1988)

Most of crosses between *met-1* and the known markers gave the map distance as > 50%, which indicates nonlinkage with *met-1* (Harris, 1989). However, the data from crosses between *met-1* and each of the following three markers: *ery2*, *maa4* and *agg1* revealed the map distance< 48%. All three of these markers had been previously mapped to the same linkage group XIV (Harris, 1989; Dutcher et al., 1991). The map distance between *met-1* and the three markers were 33 %, 43 % and 46 %. This result showed that the *met-1* mutation is located in the linkage group XIV. According to the mapping distance, *met-1* is located at the right arm of the centromere, at 33 cM to *ery2*, 43 cM to *maa4* and 46 cM to *agg1*. The map of the linkage group XIV may therefore be modified as:

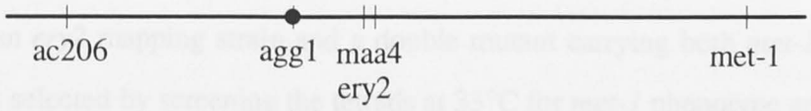


Table 3.4.1. Tetrad data from crosses between *met-1* and loci for each linkage group

Markers crossed with met-1	Linkage groups of the tested markers	Number of zygospores scored	Ratio of PD:NPD:T	Percentage recombination	Presence of linkage
ery3	I	68	13:15:40	51.5	-
act1	II	60	8: 7:45	49.2	-
ac17	III	58	5: 6:47	50.8	-
pyr1	IV	73	9:10:54	50.6	-
pf-1	V	refused to mate			
maa8	VI	53	3: 7:43	53.8	-
act2	VI	53	9:10:34	50.9	-
can1	VII	54	3: 4:47	50.9	-
Tun1	VIII	55	4: 8:43	53.6	-
Sr1	IX	53	4:15:34	60.4	-
nic13	X	66	4: 7:55	52.3	-
ery1	XI	53	9:10:34	50.9	-
gln1	XII/XIII	68	5: 6:57	50.7	-
agg1	XIV	101	18:11:72	46.5	+
maa4	XIV	115	19: 4:92	43.5	+
ery2	XIV	104	37: 2:65	33.2	+
nic1	XV	80	4 5 71	50.6	-
y1	XVI/XVII	79	5: 7:67	51.3	-
spr1	XVIII	53	3: 8:42	54.7	-
maa13	XIX	55	6: 8:41	51.8	-

To confirm the location of the *met-1* gene in linkage group XIV, three point test crosses among *met-1*, *maa4* and *ery2* were carried out. Firstly the *met-1* mutant was crossed with an *ery2* mapping strain and a double mutant carrying both *met-1* and *ery2* mutations was selected by screening the tetrads at 33°C for *met-1* phenotype and on ery. thromycin plates for *ery2* phenotype. When this *met-1*, *ery2* double mutant was crossed

with the other *maa4* mapping strain (ie, + *ery2 met-1* x *maa4* + +), the phenotypes of 356 progeny were as follows:

+	*ery2*	*met-1*	115
maa4	+	+	113
maa4	+	*met-1*	33
+	*ery2*	+	32
maa4	*ery2*	*met-1*	19
+	+	+	22
maa4	*ery2*	+	12
+	+	*met-1*	10
			356

According to the rule of three point test crosses (Suzuki et al., 1981; Fincham et al. 1979), the rarity of + *ery2* + or *met-1* + *maa4* progeny indicates that these combinations are ones that require double cross overs and therefore indicates that the *ery2* marker is between the *met-1* and *maa4* markers. This has confirmed the mapping sequence of the *met-1* and the known markers *maa4* and *ery2* that was postulated from the single crosses described above. However, a confirmation of mapping distance between each pair of markers would require screening of a larger number of tetrads than was possible in the time available.

3.4.3. Discussion

This study identified a *met-1* gene function that is necessary for cells to pass through the metaphase/anaphase transition. Genetic mapping has shown that the *met-1* gene is located in linkage group XIV at the same side of the centromere as a previously mapped marker *ery2*, and is about 32 cM away from the *ery2* marker. Mutation of the *met-1* gene caused cells to be arrested with typical metaphase morphology without initially blocking cell growth at restrictive temperature (see subsection 3.1.).

Investigation of catastrophe and execution points provided an indication that function of the *met-1* gene is indispensable from 15 h in the cell cycle, and if it is then inactivated by the restrictive temperature, the absence of its normal activity leads to irretrievable abnormal development, or catastrophe. This time is about 4 h after commitment and during the 4 h there is presumably preparation for DNA synthesis, execution of DNA synthesis, traverse of a brief G2 phase and then the establishment of prophase, all of which do not require the *met-1* gene function. After 17 h in the cell cycle sufficient of the met-1 gene function had been completed for the cell cycle to be finished and presumably by this time the metaphase execution point has been passed in the second round of mitosis.

Since suppressor gene functions have been identified by selection of suppressor mutations it is likely that the *met-1* gene product acts together with other gene products to facilitate the metaphase/anaphase transition. When a mutated suppressor gene is present together with the *met-1* mutation, successful cell division can occur at restrictive temperature. However, when this mutated suppressor gene was separated from the *met-1* mutant by Mendelian segregation, the *met-1* cells again arrested at metaphase. The *met-1* mutant may provide a useful, perhaps a unique, system for the investigation of the control mechanism of the metaphase/anaphase transition during the plant cell cycle.

It has been suggested that exit from mitosis is dependent on the completion of metaphase, which requires inactivation of the key cell division regulator $p34^{cdc2}$. This inactivation depends on both the destruction of the mitotic cyclin and the presence of $p13^{suc1}$. The diagnosis of involvement by these two proteins has been made by detecting the effects of mutation-induced changes in stability of cyclin or the effects of deleting the *suc1* gene, but details of biochemistry are not resolved and are under investigation in a number of laboratories. In *Chlamydomonas*, restriction of the *met-1* gene function causes arrest at metaphase with the persisting high $p34^{cdc2}$ kinase activity and persisting cyclin, which indicates that in the plant kingdom as well, there is a coupling of mitotic enzyme activity with mitotic cyclin levels.

The possibility that cyclin B might be abnormal in the *met-1* mutant was considered. Polymerase chain reaction and degenerate oligonucleotide probing have

yielded several cyclin-like clones from plants, including carrot, soybean (Hata et al., 1991) and alfalfa (Hirt et al., 1992). Whether these cyclins are of the A or B type is not clear (Hata et al., 1991; reviewed by Jacobs, 1992). Previous experiments in our laboratory have also identified the presence of a $p56^{cdc13}$ like protein in plants, however it has not previously been possible to investigate the relationship between the cyclin like protein and the $p34^{cdc2}$ kinase activity during the cell cycle. The good synchrony and the metaphase arrest of the *met-1* mutant has provided a good system for confirming the presence of the cyclin B like protein and its relationship to mitotic activity of $p34^{cdc2}$ in plants. Western blotting, using antibody against $p56^{cdc13}$/cyclin B from *S. pombe,* has revealed that the level of the $p56^{cdc13}$ like protein in *met-1* cells that were cultured at 21° C increased sharply at mitosis and then declined sharply as cells completed mitosis. However in *met-1* cells at 33°C which entered mitosis and became arrested in metaphase, the $p56^{cdc13}$ like protein remained at high levels. This evidence that a protein cross reacting with antibody against $p56^{cdc13}$ is at high level coincidently with persisting $p34^{cdc2}$ activity at mitosis is probably the best currently available evidence that the plant cells contains mitotic cyclin protein and that its level is tightly linked with and may play a part in determining $p34^{cdc2}$ kinase activity.

The coincidence of the high level of cyclin B like protein and high level of $p34^{cdc2}$ kinase activity invites inquiry as to whether the failure of inactivating the $p34^{cdc2}$ kinase (thus to exit from mitosis) is due to nondegradable cyclin protein. This possibility has been eliminated by inducing mitosis in higher plant cells that were in early pre-prophase and would normally have stayed in that phase for hours, or reversed into interphase. When the active MPF was purified from the *met-1* arrested cells and microinjected into such higher plant cells, metaphase was induced and progressed normally to anaphase. The induction of higher plant cell division by microinjection of purified plant MPF has not been previously reported. The result from this experiment not only provided evidence that the MPF from the unicellular plant is competent to initiate higher plant mitosis but also indicates that the cyclin protein in the MPF from arrested *met-1* cells is degradable. Therefore arrest is not due to an inherent non-degradability of the cyclin of *met-1* cells.

The successful degradation of the mitotic cyclin of *met-1* cells at permissive temperature and the presumed inactivation of *met-1* MPF in higher plant cells as they progress into anaphase suggests that the gene product that is altered by the *met-1* mutation might be involved in a function that is either involved in signalling the appropriateness of inactivation of $p34^{cdc2}$ kinase when metaphase has been established or is directly involved in the process of $p34^{cdc2}$ inactivation.

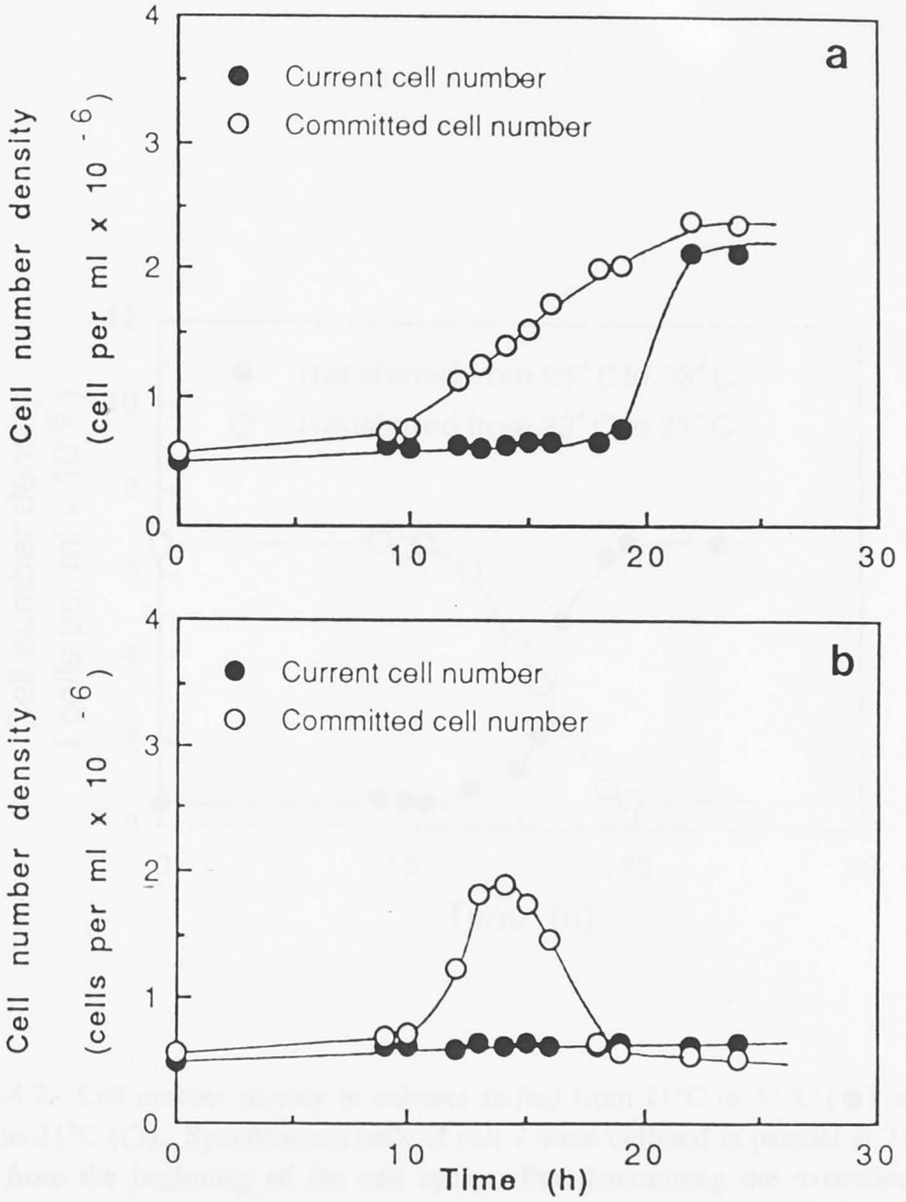


Fig. 3.4.1. Current cell number and committed cell number in synchronous cultures of the metaphase-arresting mutant (*met-1*) at 21°C and 33°C. Synchronous cells from the same parent culture were inoculated in parallel and grown from the beginning of the cell cycle at 0 h, at both 21°C and 33°C. For measurement of the current cell number (closed circles), samples were taken at the times plotted and fixed with 1% formaldehyde. The cell number was counted with a Coulter counter Model ZB (Coulter electronic, Pty., Ltd., NSW 2100, Australia). For measurement of committed cell number (open circles), samples were taken at times plotted and transferred to total darkness at 21°C, cell number was measured with the Coulter counter at a time when all committed divisions were complete. (a), current cell number and committed cell number in synchronous culture at 21°C; (b), current cell number and committed cell number in synchronous culture at 33°C.

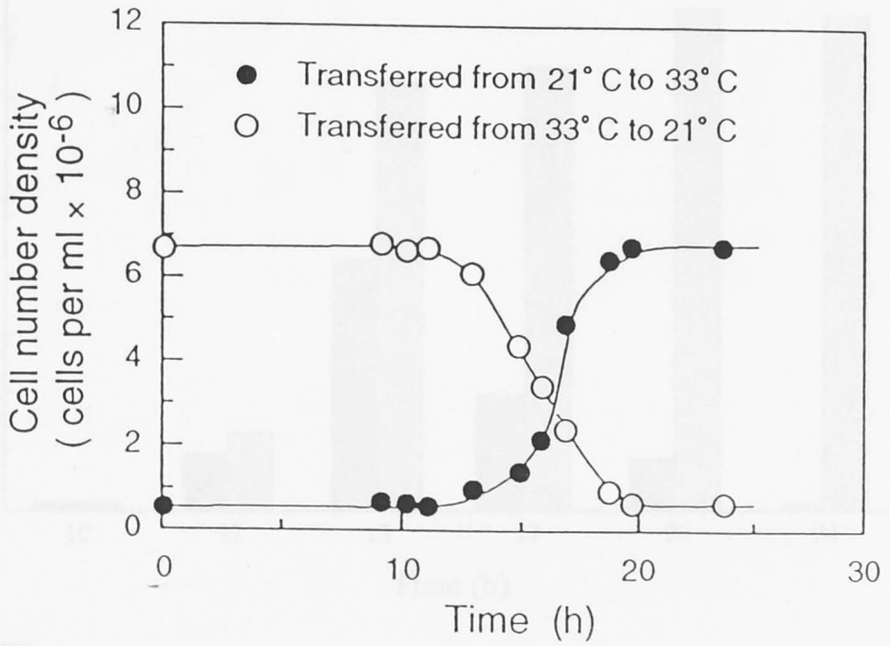


Fig. 3.4.2. Cell number density in cultures shifted from 21°C to 33°C (●) and from 33°C to 21°C (○). Synchronous cells of *met-1* were cultured in parallel at 21°C and 33°C from the beginning of the cell cycle. For determining the execution point, samples taken at times plotted were transferred from 21°C to 33°C, cell number in each shifted subculture was measured after one cell cycle duration and plotted at the time of transfer (closed circles). For determining the catastrophe point, samples taken at times plotted were transferred from 33°C to 21°C, cell number in each transferred subculture was measured at a time when divisions that the cells were competent to perform were complete (48 h after the beginning of the cell cycle) and is plotted at time transferred (open circles). Cultures and transferred subcultures were illuminated throughout.

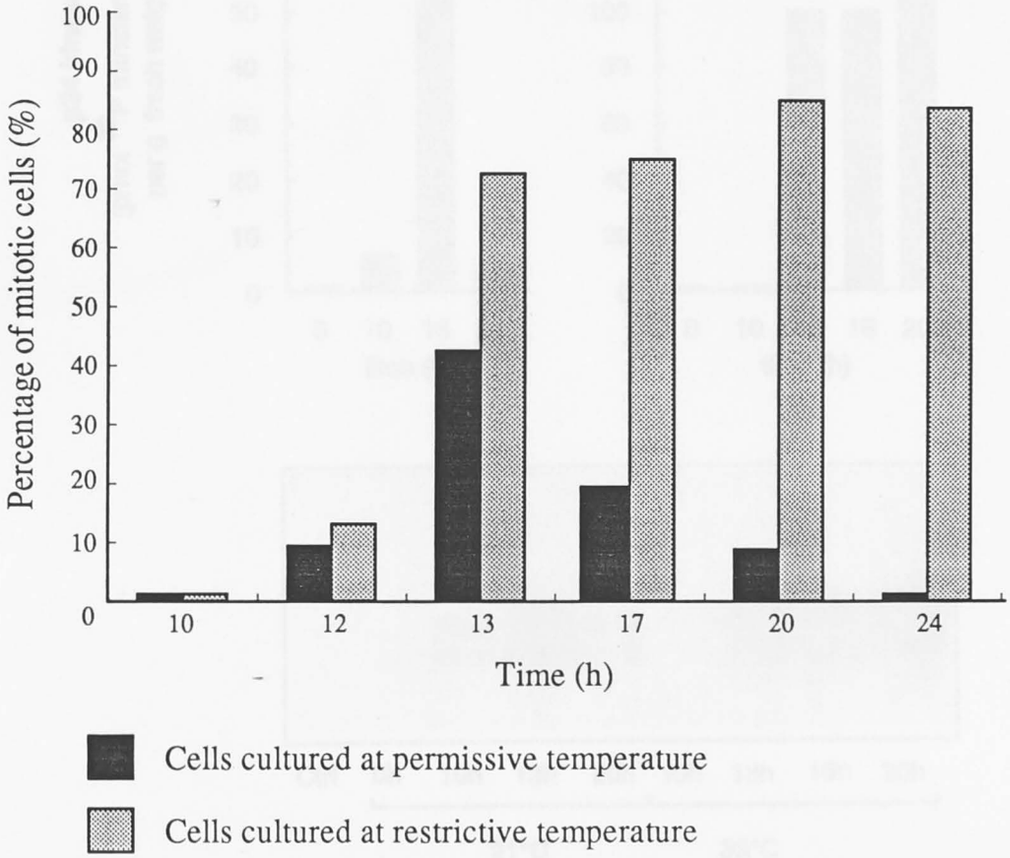


Fig. 3.4.3. Mitotic index in synchronous *met-1* cultures that were taken from the same synchronous inoculum at the beginning of the cell cycle and grown at the permissive and restrictive tempratures. Samples were taken from both cultures at the times plotted. Fixed cells were stained with DAPI to determine the percentage of cells with mitotic figures.

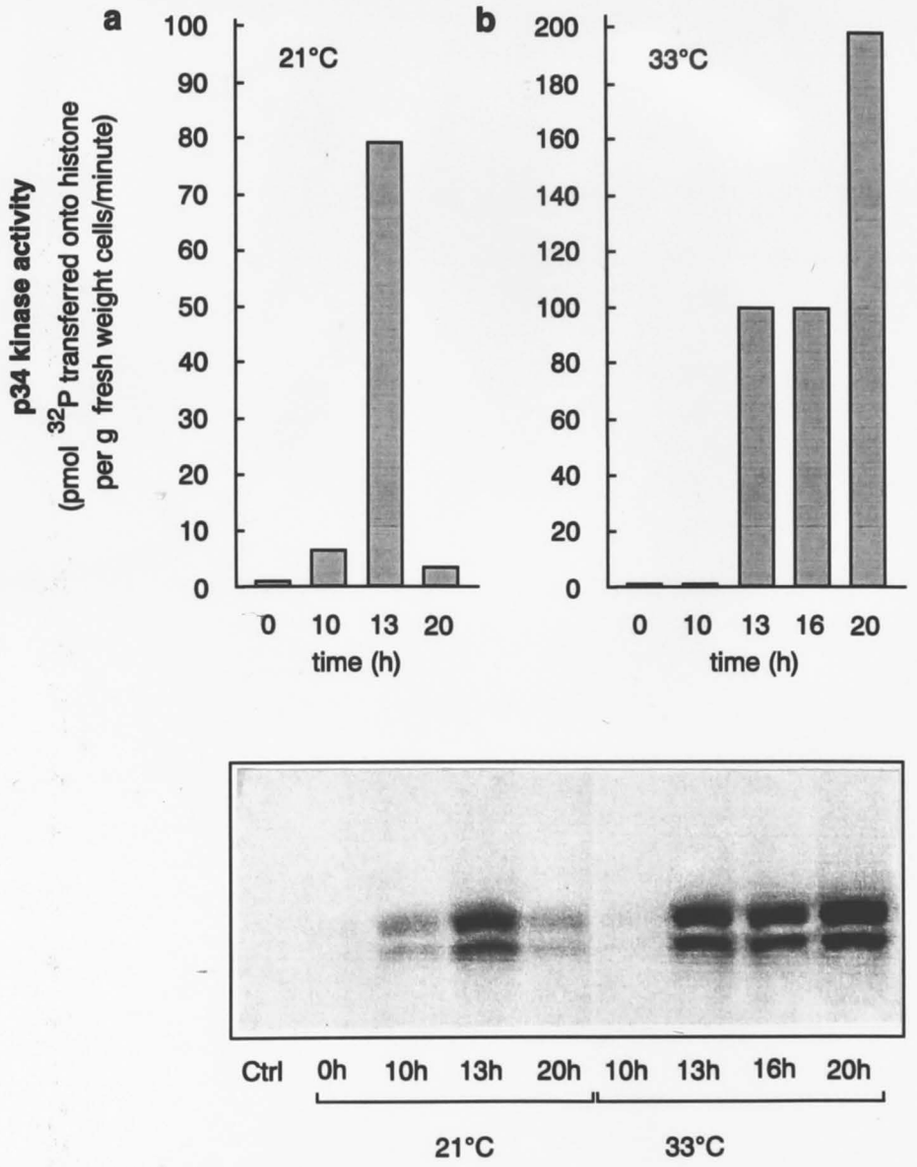
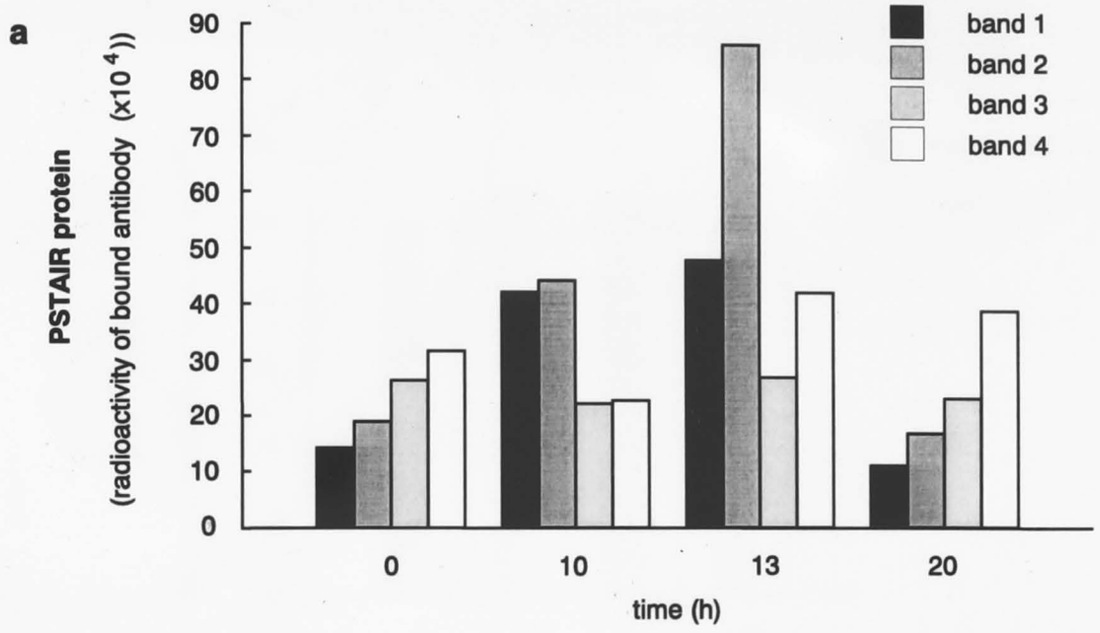
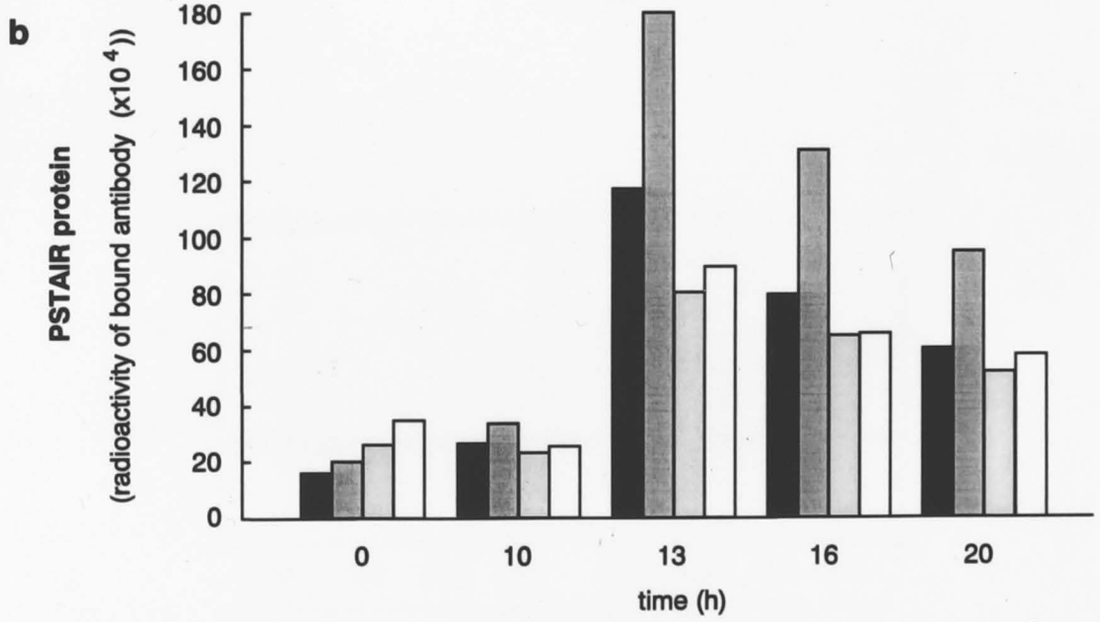
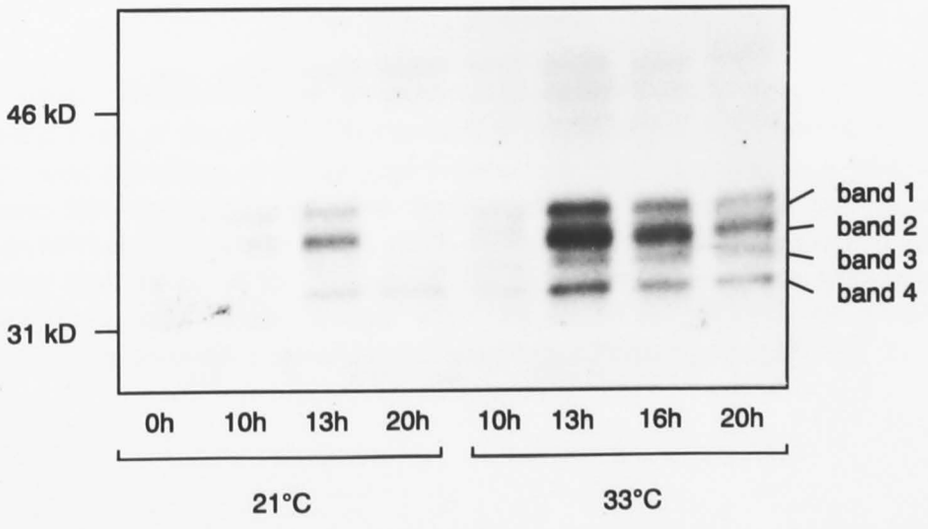


Fig. 3.4.4. Effect of the *met-1* mutation on $p34^{cdc2}$-like H1 kinase activity during the cell cycle. Synchronous cells of *met-1* were cultured in parallel at 21°C (a) and 33°C (b) from the beginning of the cell cycle. Samples were taken from both cultures at times indicated. For measurement of the activity of $p34^{cdc2}$-like protein kinase, 0.01g fresh weight of cells ground in liquid nitrogen were extracted in NDE buffer (see general methods), the $p34^{cdc2}$ like protein kinase was purified from each sample by binding the kinase to 20 µl of $p13^{suc1}$ beads,two washes with detergent buffer and one wash without detergent,then eluting with 50 µl of 0.5 mg/ml of free $p13^{suc1}$ solution. The activity of purified $p34^{cdc2}$-like protein kinase was measured using histone H1 as a substrate at 30° C for 5 min. The activity is plotted as the amount of ^{32}P transferred onto histone H1, which was measured by placing 20 µl of reaction mixture on P81 phosphocellulose paper, washing with phosphoric acid and then counting in a scintillation counter. The radioactivity of the reaction product, histone H1, shown in the panel at the bottom of the figure, was obtained by separation of 30 µl of reaction mixture on a 12% acrylamide gel and then exposure in a PhosphorImager. Note that the ordinate scale is smaller in (a).

Fig. 3.4.5. Effect of the *met-1* mutation on levels of the p34^{cdc2}-like proteins during the cell cycle. Equal loadings of 50 µg total protein, extracted from synchronous *met-1* cells cultured at 21°C (a) and 33°C (b), were separated on a 10-15% linear gradient SDS-acrylamide gel. A Western blot of transferred proteins was probed with anti PSTAIR antibody and the bound antibody was detected by ^{125}I anti-rabbit IgG. The Western blot with ^{125}I labelled p34^{cdc2}-like proteins, shown at the bottom of the figure, was obtained by exposure of the nitrocellulose onto Kodak X-omat film. Levels of the four bands of p34^{cdc2}-like protein were determined by quantification of the bound isotope on a PhosphorImager. Note the difference in ordinate scale in (a) and (b).


a
PSTAIR protein
(radioactivity of bound antibody (x10 4))
90
80
70
60
50
40
30
20
10
0
band 1
band 2
band 3
band 4
0
10
13
20
time (h)


b
PSTAIR protein
(radioactivity of bound antibody (x10 4))
180
160
140
120
100
80
60
40
20
0
0
10
13
16
20
time (h)


46 kD
31 kD
band 1
band 2
band 3
band 4
0h
10h
13h
20h
10h
13h
16h
20h
21°C
33°C

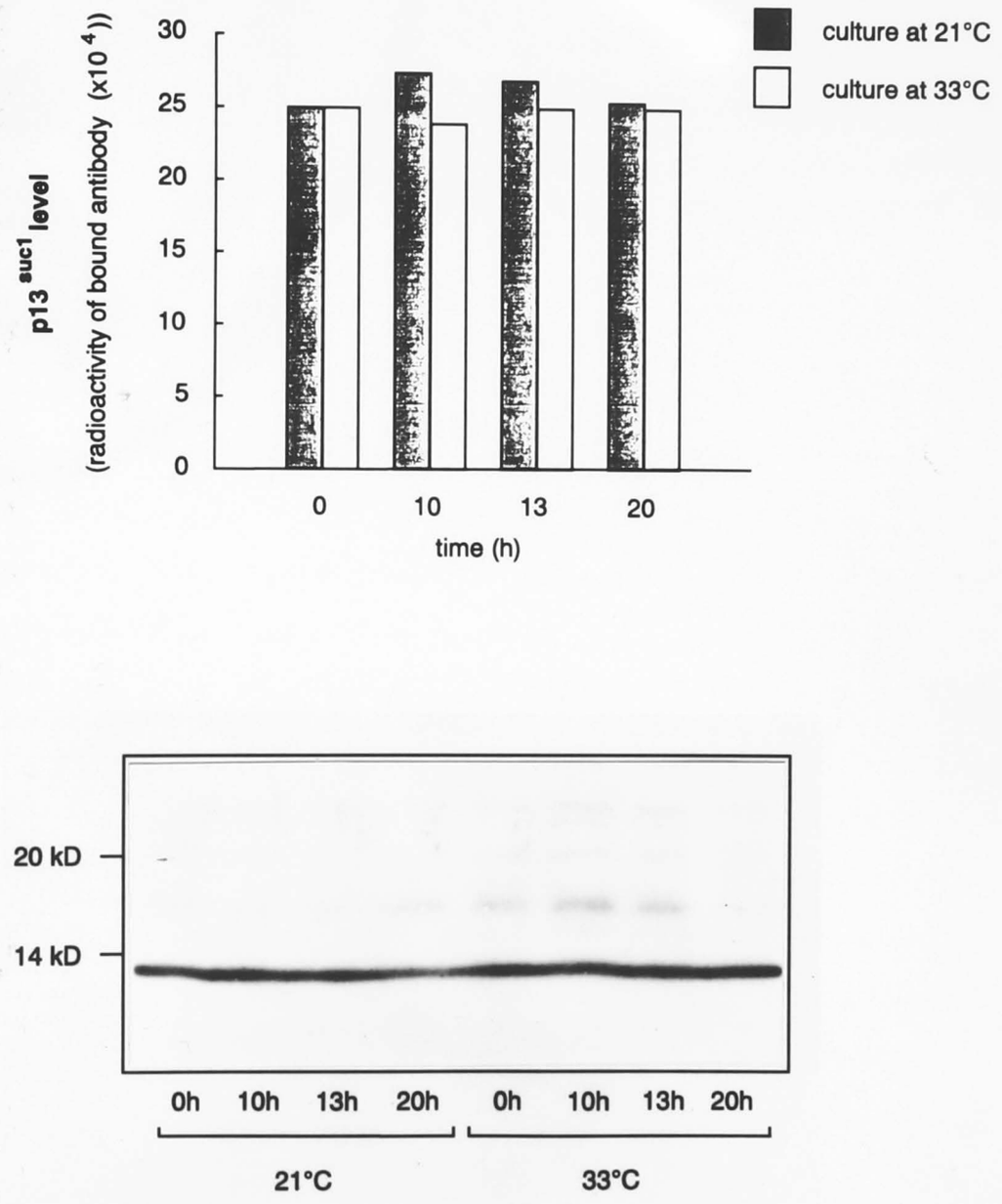


Fig. 3.4.6. Immunoblotting of extracts from *met-1* with anti-p13^{suc1} antibody, showing levels of the p13^{suc1}-like protein in synchronous cells cultured at 21°C and 33°C. Equal loadings of 50 µg total protein that were separated on a 10-25% linear gradient SDS-acrylamide gel were transferred onto nitrocellulose and probed with anti-p13^{suc1} antibody. The bound antibody was detected by ^{125}I anti-rabbit IgG. The Western blot shown at the bottom of the figure was obtained by exposure of the nitrocellulose onto Kodak X-omat film. Levels of the p13^{suc1}-like protein were determined by quantification of bound isotope on a PhosphorImager.

Fig. 3.4.7. Correlation of (a) levels of a cyclin B-like protein with (b) activity of affinity purified $p34^{cdc2}$-like protein kinase in synchronous cultures of the *met-1* mutant that arrests in metaphase, seen in (c) by β-tubulin staining of the spindle and (d) DAPI staining of the aligned condensed chromosome (same cell), the arrested phenotype being held indefinitely e.g. in (e) at 20 h, β-tubulin stained. Magnification: (c) (d) x5000; (e) x3000. At the permissive temperature of 21°C cells divided normally with high mitotic activity at 13 h terminating by 20 h when cells were in cytokinesis. The levels of cyclin-B like protein, detected by anti-$p56^{cdc13}$ antibody on Western blot, and $p34^{cdc2}$-like protein kinase purified by $p13^{suc1}$ binding, then elution with free $p13^{suc1}$, and assayed with H1 histone, shown after electrophoresis and autoradiographic detection, are both detected only in mitotic cells at 13 h. In cells grown at the restrictive temperature of 33° C, cyclin B-like protein and $p34^{cdc2}$-like kinase activity appeared in mitotic cells but the persistence of cyclin correlates with continued activity of the kinase and inability to progress beyond metaphase.

Chlamydomonas *met1 cdc* mutant synchronous culture

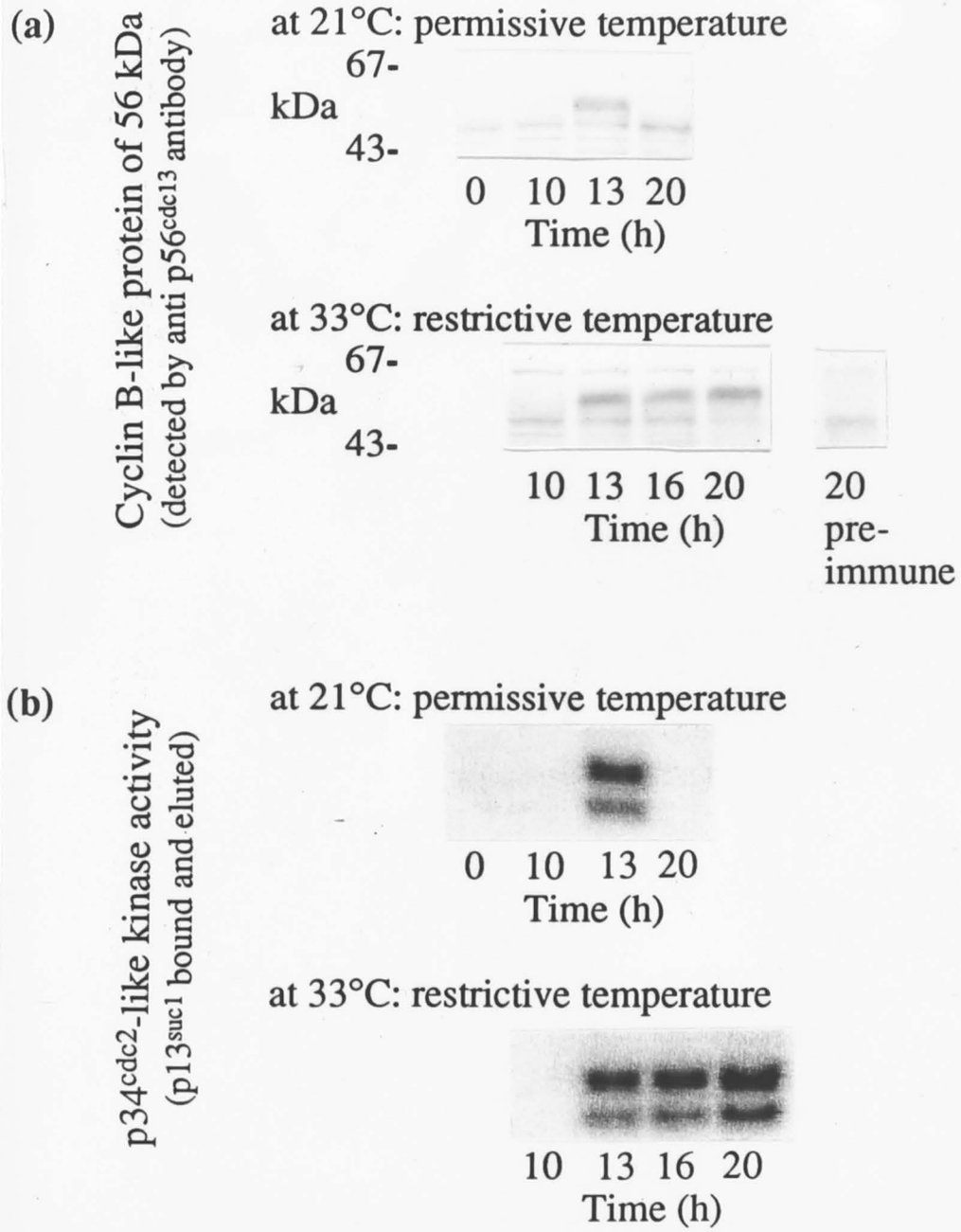


Fig. 3.4.8. Time course of mitosis following microinjection of *Chlamydomonas* MPF from arrested *met-1* cells into stamen hair cell of *Tradescantia virginiana*.

(a), immediately after injection.

(b), (c), (d), (e), [4, 5, 13, and 18 min after injection]; premature chromosome condensation is seen with the chromosomes becoming more condensed than is usual prior to nuclear envelope breakdown (NEB). Partial NEB is established close to the site of injection.

(f), [33 min after injection]; complete NEB, establishing prometaphase.

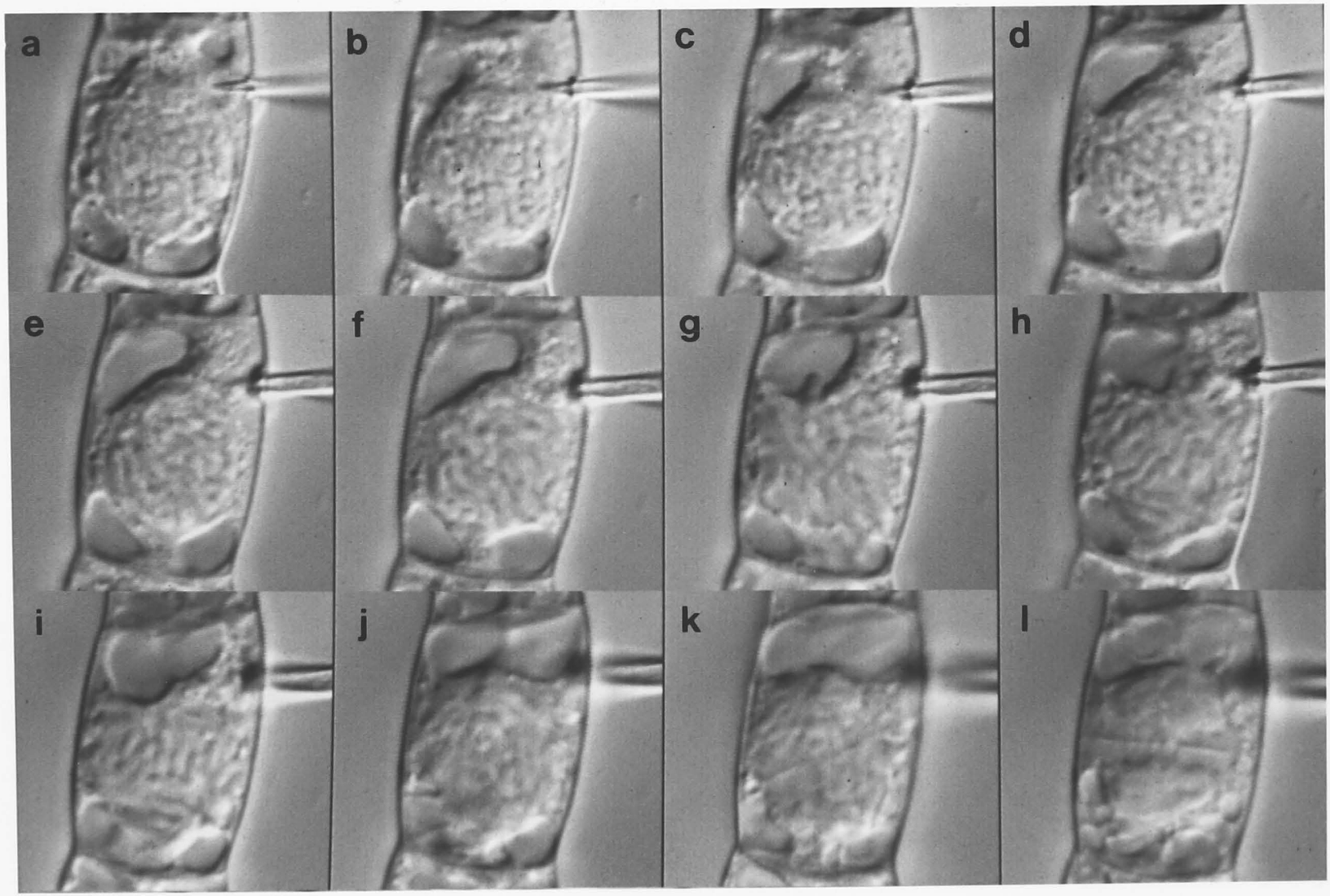
(g), (64 min after injection]; metaphase.

(h), [77 min after injection]; early anaphase.

(i), (j), [88, 99 min after injection]; mid anaphase.

(k), [110 min after injection]; telophase and early cell plate formation.

(l), [127 min after injection]; phragmoplast almost completely formed.


a
b
c
d
e
f
g
h
i
j
k
l

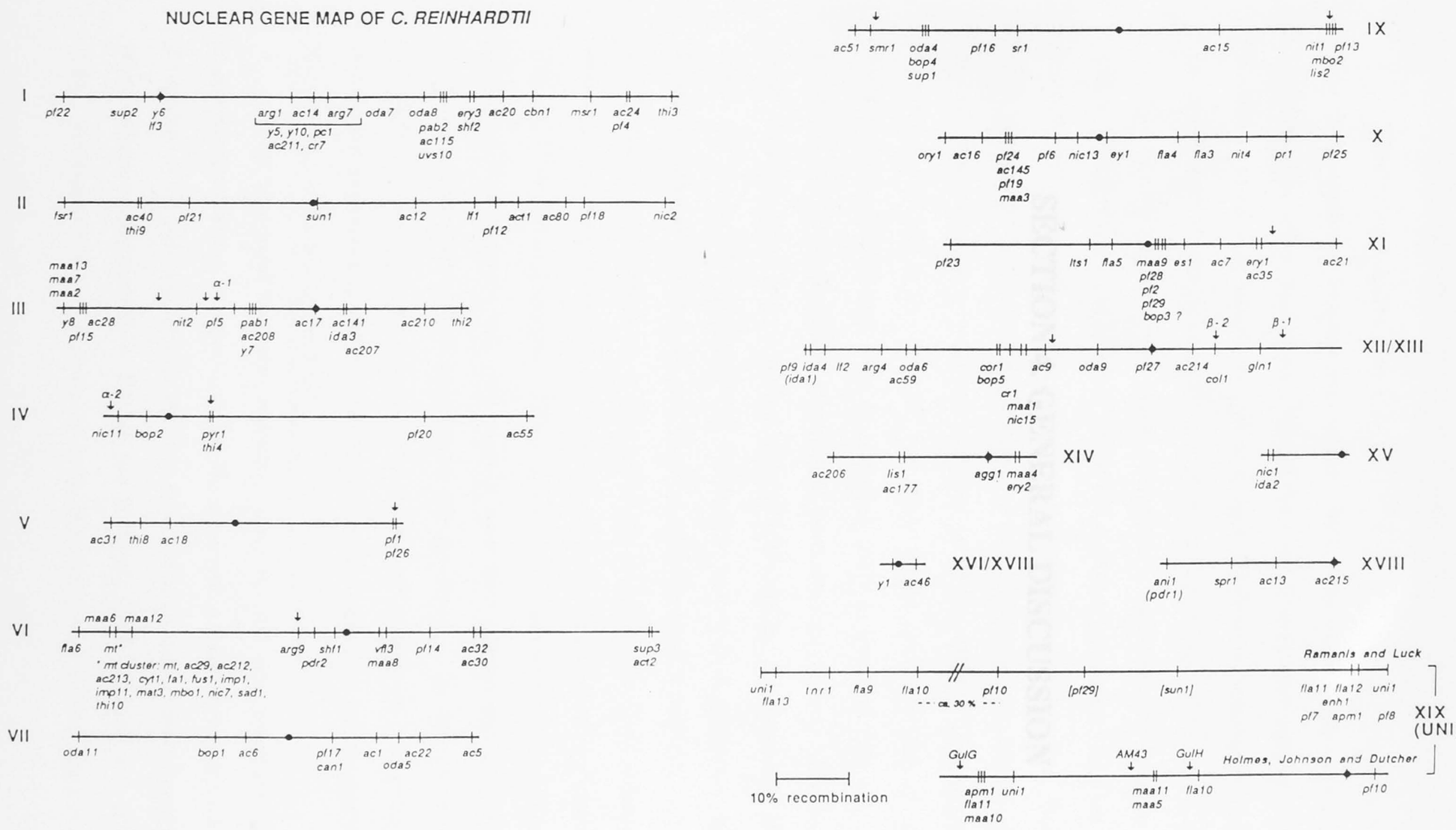


Fig. 3.4.9. The nuclear gene map of *Chlamydomonas reinhardtii*. (from Harris,1989)

SECTION 4. GENERAL DISCUSSION

Chlamydomonas has long been used as a model system for genetic analysis of biological phenomena (Holmes and Dutcher, 1992). The work described in this thesis has focused on using this cell for study of the plant division cycle and has shown its advantages in allowing isolation of a range of plant cell division cycle (cdc) mutants that are conditionally blocked in either G1, G2, M or cytokinesis phase of the cell cycle.

An early attempt to isolate temperature sensitive cdc mutants from *Chlamydomonas* was made by Wolff (1971) whose PhD study included isolation of four temperature sensitive mutants that could be partially arrested in cell division. Pioneering work that resulted in isolation of a collection of temperature sensitive cell division cycle mutants was carried out by Howell and Naliboff (1973, 1974). Unfortunately the techniques available at the time did not allow speedy characterisation of terminal phenotypes and the mutants were characterised solely in terms of their cell number increase in asynchronous cultures after shift to restrictive temperature. After the period when they were being actively studied some 20 years ago the maintenance of Howell's stocks has not included procedures to discriminate against back mutations, therefore Howell's mutant collection is of very doubtful value for present research. Since the 1980s, efforts have been made in our laboratory to isolate plant cell division cycle mutants from *Chlamydomonas* and some of the mutants have been partially characterised (Harper, 1986; Sakuangrunsirikul, 1991). Up to now, a collection of 51 temperature sensitive *Chlamydomonas* cell cycle mutants have been isolated and maintained by careful selection against back mutation in our laboratory. In the present study, investigation of some of these *Chlamydomonas* cell cycle mutants has been extended to include immunomicroscopy of cytoskeletal and nuclear structures and the analysis of nuclear DNA contents in arrested cells.

It has been found by genetic analysis of yeasts that cell cycle events are initiated in dependent sequences, in which initiation of a late event in a sequence is dependent on completion of the previous events in the same sequence but is independent of progress in other sequences (Hartwell,1974; Pringle and Hartwell, 1981; Hartwell and Weinert, 1989). An example of such dependency is the observation that without completion of

DNA synthesis normal cells do not initiate mitosis (Enoch and Nurse, 1990; Li and Murray, 1991; Smythe and Newport, 1992). It has been observed in yeast that more than one dependent sequence can be occurring in parallel, for example budding and nuclear division sequences diverge after START of division.

In *Chlamydomonas*, newly divided wild-type cells growing at moderate to fast rate under laboratory conditions, which usually allow more than fourfold increase in mass in 14 h, normally spend 9 to 11 h in G1 prior to the attainment of commitment to divide. The duration of this period is determined by a biological timer since it is compensated against changes of temperature in the range 20°C to 30°C and is insensitive to cell size. During the timed period cells that are growing at all but the very slowest rates become big enough to produce more than two daughter cells. Cells must be growing to attain commitment to divide but thereafter proceed without requirement for further growth into nuclear DNA replication, then through a very brief G2 phase to mitosis and cytokinesis (Donnan and John, 1983; Donnan et al., 1985).

A similar divergence of cytokinesis and nuclear division sequences has been observed to follow the commitment to division control point in late G1 phase of *Chlamydomonas*. Parallel dependent sequences of cell cycle events have also been identified in *Chlamydomonas* (Harper and John, 1986; John, 1987). Progress to attempted cytokinesis is at least in part independent of nuclear division (Harper and John, 1986; Harris, 1989), since wild type *Chlamydomonas* cells that are blocked in S phase by inhibition with hydroxyurea or 2-deoxyadenosine can initiate cytokinesis even though no nuclear division has occurred because of the block (Harper and John, 1986). This result indicates that although cytokinesis normally occurs after cells have completed nuclear division, preparation for this event may start much earlier than the time when it is actually visualised and prior to completion of S phase. It is deduced that initiation of cytokinesis occurs after the attainment of commitment to divide and before the completion of DNA replication. A similar example of preparation for cytokinesis beginning soon after commitment is provided by the diversion of acetate carbon into future components of the sporopollenin layer that forms around daughter cells in the unicellular alga *Chlorella* (John et al., 1973). The sporopollenin that is assembled at the

end of the cycle is most abundantly labelled with ^{14}C acetate when this is provided in G1 phase soon after commitment and long before daughter formation. At this time in G1 sporopollenin precursors are presumably being synthesised, although the actual polymerisation of the new cell wall layer only begins 8 h later after cytokinesis (John et al., 1973).

The present study has supported the conclusion that progress to cytokinesis can occur in partial independence of progress in the DNA-replication-nuclear-division sequence. It has also been detected that a checkpoint that is apparently initiated in G2 phase couples initiation of the cleavage furrow to the prior successful attainment of metaphase. This checkpoint will be discussed below, after consideration of the main features of the mutants.

The G1-arresting mutant *cdG1-1*, although not far advanced in the DNA-division sequence when arrested, evidently contained a cell cycle type of mutation. At the restrictive temperature it was not blocked in growth since it could attain an average cell size that was six or seven times the average daughter cell size (Fig. 3.1.1). The attainment of commitment to divide was also detectable under restrictive condition (Fig. 3.2.1) and the arrest phenotype indicated that flagella regression had also been attained (Fig. 3.1.11), although DNA duplication did not occur. There was however no indication that other cytoskeletal depolymerisation events were coupled to flagella regression since no detectable modification of the cortical cytoskeleton was seen in this arrested mutant. In conformity with this conclusion the accompanying analysis of the G2-arresting mutants identified depolymerisation of the cortical microtubules as being a separate G2 event. The early initiation of flagella regression prior to DNA duplication, which is suggested by the arrest phenotype of the G1 mutant, was also indicated by temporal measurements of flagella regression in wild type cells. These showed that regression began about 2 h after first commitment (Fig. 3.2.4) and is therefore approximately concurrent with the G1/S transition (Donnan and John, 1983).

Defects in G2 functions caused by the *cdM-1* and *cdM-2* mutations resulted in arrest at mitotic initiation with doubled nuclear DNA content but with interphase-like cortical microtubules. The conclusion that the block in these mutants is in late G2, at

mitotic initiation, rests upon the occurrence of a partial activation of the $p34^{cdc2}$ kinase (Fig. 3.3.7 and Fig. 3.3.8) that normally immediately precedes mitosis (Fig. 3.3.6). The blockages caused by *cdM-1* and *cdM-2* mutations not only prevented depolymerisation of the cortical microtubules but also mitotic spindle formation, indicating that the two microtubule configurations, cortical or spindle, may be mutually exclusive; perhaps because they are dependent on alternative activation states of $p34^{cdc2}$ kinase, or because formation of the spindle requires tubulin monomer that may have to be derived from the cortical cytoskeleton.

In higher plants, depolymerisation of the cortical microtubules and formation of the preprophase band (PPB) is an early structural indication of the approach of mitosis (Wick and Duniec, 1983). This shift of cortical microtubules to the PPB begins on completion of DNA replication, progresses throughout G2 phase and is complete at preprophase (Gunning and Sammut, 1990). In *Chlamydomonas*, however, changes of microtubule-based structures prior to mitosis include both regression of flagella beginning in late G1 phase and depolymerisation of the interphase cortical microtubules (Doonan and Greif, 1987). Because of the brief G2 phase in *Chlamydomonas*, it has previously been difficult to identify cell cycle events that occur specifically in G2 phase. Observations of synchronous wild type cells and of the cell division mutants in the present work have revealed that regression of flagellar and depolymerisation of interphase cortical microtubules are two separately initiated events. Regression of flagella is initiated soon after commitment in late G1 phase near the G1/S transition and is completed in G2 phase about 0.6 h before the first prophase of the cell cycle (Fig. 3.2.4). Whereas depolymerisation of the interphase cortical microtubules was identified as a G2/M event occurring after the block point of *cdM-1* and *cdM-2* and thereafter at mitotic initiation.

The conservation of mitotic initiation as the switch that causes the change from cortical to nuclear microtubule deployment in both *Chlamydomonas* and higher plants is in accord with the proposition that the key molecular event is the activation of $p34^{cdc2}$ kinase (John and Wu, 1992; John et al., 1993a,b). This hypothesis is further supported by the observation that the G2-arresting mutants that partly activate the $p34^{cdc2}$ kinase

are unable to effect the cytoskeletal switch. An implication of the conservation of timing and possible mechanism of cytoskeletal change between the unicellular and higher green plants is that the unicellular plant provides a model system for genetic analysis that can be of extensive relevance.

The occurrence or otherwise of attempted cytoplasmic cleavage in the arrested mutants and in wild type cells indicates a possible check point that is established in G2 phase and couples initiation of the cleavage furrow to successful attainment of metaphase. It has earlier been postulated that the initial impetus for cytokinesis comes from commitment to division or soon after that since cells blocked in DNA duplication by hydroxyurea or 2-deoxyadenosine do attempt cytokinesis (Harper and John, 1986). The point of initiation can now be pin-pointed more specifically from evidence that the G1 arresting *cdG1-1* cells are unable to initiate cytokinesis. Therefore attainment of commitment, which can be detected in *cdG1-1* (Fig. 3.2.1), is not the point of initiation of the cytokinesis pathway, rather very late G1 phase or early S phase is indicated as the time of initiation of progress to cytokinesis. Although cells chemically blocked in DNA synthesis can proceed to attempted cytokinesis, cells arrested in G2 phase by the *cdM-1* or *cdM-2* mutation cannot. This suggest that a check point is established in G2 phase that requires progress in mitosis before cytokinesis can be initiated (Fig. 4.1). Chemicals that block DNA synthesis may therefore evade this checkpoint by not allowing cells to enter G2 phase although having passed commitment. This circumstance of failure to enter G2 phase would not occur naturally since even starved cells will progress through S and G2 phases once committed to division, therefore the checkpoint will not often be avoided. The extent of progress in mitosis that is required to satisfy the checkpoint can be deduced from the fact that it cannot be fulfilled by oryzalin inhibited cells and therefore basal body duplication and separation, which occurred in the presence of oryzalin, are insufficient to allow cytokinesis. However the arrest point of the *met-1* mutation is sufficient to allow cytokinesis, therefore formation of a spindle and attainment of metaphase is sufficient to satisfy the check and actual completion of nuclear division is not required.

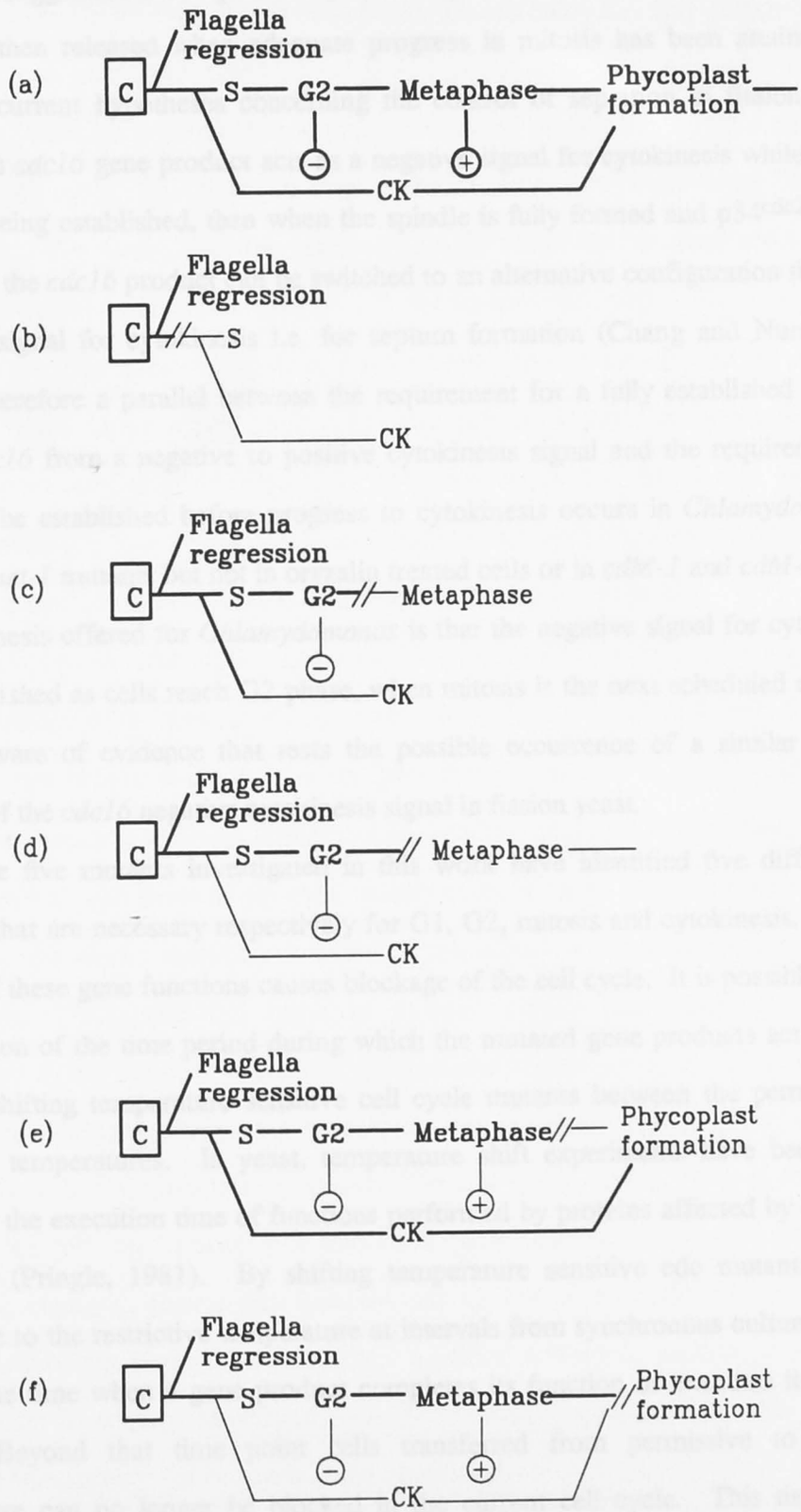


Fig. 4.1. Dependency relationships that can account for arrest configurations in *Chlamydomonas*. (a), wild type; (b), *cdG1-1*; (c), *cdM-1* and *cdM-2*; (d), oryzalin treated wild type cells; (e), *met-1*; (f), *cdCK-1*. Description in boldface: events can complete; description (nonbold): blocked events.

The suggestion that a potentially inhibitory state for cytokinesis can be set up in G2 phase, then released when adequate progress in mitosis has been attained, has a parallel in current hypotheses concerning the control of septation in fission yeast by *cdc16*. The *cdc16* gene product acts as a negative signal for cytokinesis while a mitotic spindle is being established, then when the spindle is fully formed and $p34^{cdc2}$ kinase is fully active the *cdc16* product can be switched to an alternative configuration that acts as a positive signal for cytokinesis i.e. for septum formation (Chang and Nurse, 1993). There is therefore a parallel between the requirement for a fully established spindle to change *cdc16* from a negative to positive cytokinesis signal and the requirement for a spindle to be established before progress to cytokinesis occurs in *Chlamydomonas*, as occurs in *met-1* mutants but not in oryzalin treated cells or in *cdM-1* and *cdM-2* mutants. The hypothesis offered for *Chlamydomonas* is that the negative signal for cytokinesis is only established as cells reach G2 phase, when mitosis is the next scheduled event. We are not aware of evidence that tests the possible occurrence of a similar G2 phase initiation of the *cdc16* negative cytokinesis signal in fission yeast.

The five mutants investigated in this work have identified five different gene functions that are necessary respectively for G1, G2, mitosis and cytokinesis. Failure in any one of these gene functions causes blockage of the cell cycle. It is possible to obtain an indication of the time period during which the mutated gene products act in the cell cycle by shifting temperature sensitive cell cycle mutants between the permissive and restrictive temperatures. In yeast, temperature shift experiments have been used to determine the execution time of functions performed by proteins affected by conditional mutations (Pringle, 1981). By shifting temperature sensitive cdc mutants from the permissive to the restrictive temperature at intervals from synchronous cultures, one can identify the time when a gene product completes its function or executes its cell cycle event. Beyond that time point cells transferred from permissive to restrictive temperature can no longer be blocked in the current cell cycle. This time point is designated the "execution point". However, the execution point provides no information concerning when a cell cycle gene may have begun to make its contribution to the cell cycle.

Temperature shifting experiments in the present study not only allowed detection of the execution point, but also allowed identification of a time point after which cells that were shifted back from the restrictive to permissive temperatures could not complete the current cell cycle. This time point has been designated the "catastrophe point" of the cdc mutants in this study, because incubation at the restrictive temperature beyond this time point causes irreversible failure of subsequent division. By measuring the catastrophe and the execution points, a time period can be determined for a particular cell cycle mutant during which the mutated gene product is most likely to make its major contribution to the cell cycle (Fig. 4.2). It is most likely that the normal gene product begins to function just before or at the catastrophe point and that sufficient of the function for the current cell cycle is completed at the execution point.

However the catastrophe and execution points can provide only a guide to the times of function of the gene products. Function may have begun a little earlier than the time when catastrophe is detected if the early absence of normal contribution produces only a mild distortion of progress that can be retrieved at the permissive temperature. In some organisms with some single gene mutations catastrophe may not develop at all. In *Aspergillus* the *nimA5* mutation that blocks preceding the initiation of mitosis (Oakley and Morris, 1983) allows a burst of mitotic activity upon transfer to permissive temperature by all the cells that were accumulated at the arrest point but were not in catastrophe. The reversibility seen with *nimA5* may result because the *nimA* functions at the main switch for initiation of mitosis (Osmani et al., 1987; Osmani et al., 1991) therefore the cell may remain in a stable G2 configuration with no ~~unbalanced~~ partly completion changes. Conversely function may also be completed a little later than the apparent execution point indicates if the modified cell cycle protein only slowly or partially loses its activity, in which case sufficient activity may continue after the shift to restrictive temperature for the cycle to be completed.

The relative timing of transition points for cdc gene functions within the DNA-division sequence is of interest. Therefore these have been calculated relative to the START of the DNA-division sequence (Table 3.3.1). On this basis the results for different genes in different experiments are comparable especially since the post

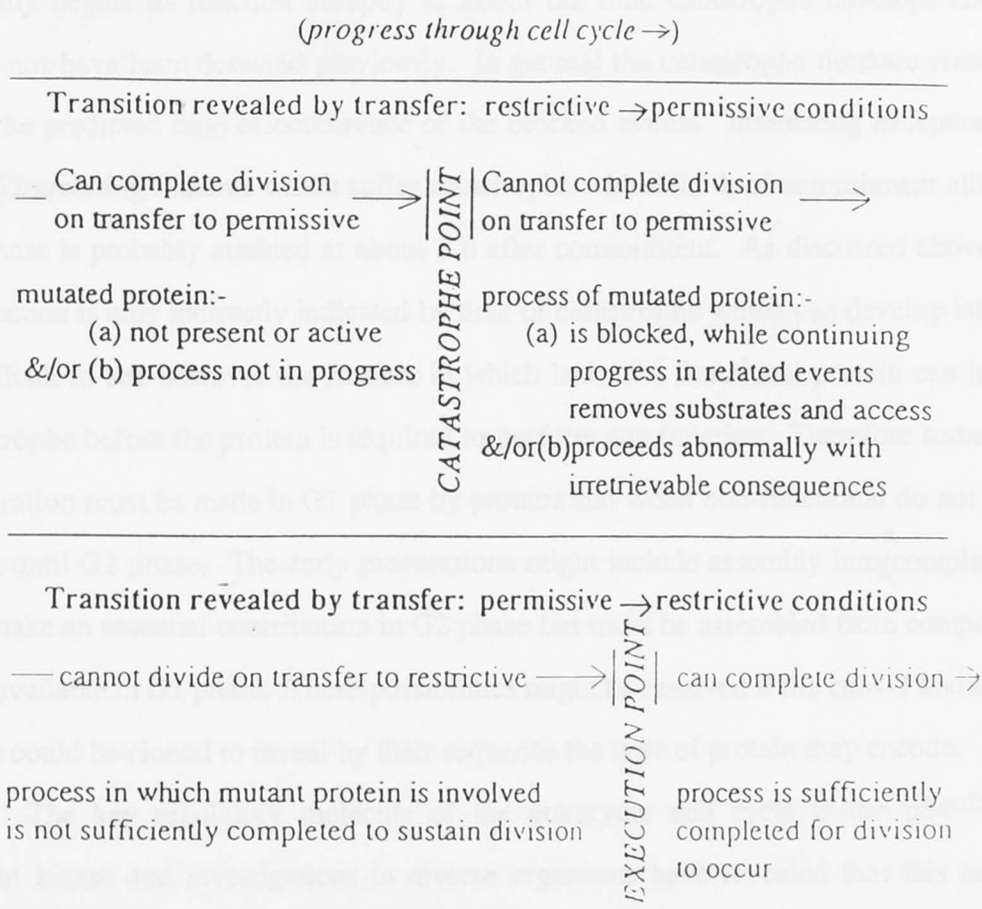


Fig. 4.2. Transitions in cdc gene function revealed by temperature shift.

commitment phase, equivalent to the DNA-division sequence events, is of a relatively stable 6 h over a range of growth rates between mass doubling time of 5 to 25 h (Donnan and John, 1983).

The existence of abrupt transitions to a state of catastrophe not long before the block points of the mutants is of interest since this form of experiment has not been extensively reported in other organisms. A likely conclusion is that the affected protein normally begins its function abruptly at about the time catastrophe develops and this could not have been detected previously. In general the catastrophe times are consistent with the predicted time of occurrence of the blocked events. Interesting exceptions are two G2 arresting mutants which suffer catastrophe within 0.5 h of commitment although G2 phase is probably attained at about 3 h after commitment. As discussed above time of function is only indirectly indicated by time of catastrophe which can develop later. It is difficult to see however the manner in which lack of a functional protein can lead to catastrophe before the protein is required to perform any function. Therefore some early preparation must be made in G1 phase by proteins that when non-functional do not cause arrest until G2 phase. The early preparations might include assembly into a complex that will make an essential contribution in G2 phase but must be assembled from components only available in G1 phase. These possibilities might be resolved if the *cdM-1* and *cdM-2* genes could be cloned to reveal by their sequence the type of protein they encode.

The key regulatory molecule of the eukaryote cell cycle is the $p34^{cdc2}$-like protein kinase and investigations in diverse organisms have revealed that this enzyme constitutes the catalytic subunit of a mitosis (or maturation)-promoting factor (MPF) which is a physical complex of $p34^{cdc2}$ with cyclin B (reviewed by Nurse, 1990). Immunoblotting with anti PSTAIR antibody in the present study allowed detection of four $p34^{cdc2}$-like protein bands with molecular weights of 38, 36, 34 and 32 kDa. Detection of four PSTAIR-containing protein bands, instead of three as reported by John and co-workers (1989), might have resulted from use of a gradient gel. It is possible that the four $p34^{cdc2}$-like protein bands represent different phosphorylation forms of $p34^{cdc2}$ which act at different stages of the cell cycle. It is also possible that they represent different variants of $p34^{cdc2}$-like protein kinase, possibly including a cdk2-like gene

product as in other organisms (Tsai et al., 1991; Meyerson et al., 1992; Peeper et al., 1993). *Chlamydomonas* has a complex cell division cycle involving multiple fission that may require a more complex regulatory machinery including variants of cdc2.

Levels of $p34^{cdc2}$ protein kinase are constant during the cell cycle of most eukaryotes that have been investigated, but the kinase activity of $p34^{cdc2}$ changes with the cell division cycle (Draetta and Beach, 1989). Although the presence in *Chlamydomonas* of proteins resembling $p34^{cdc2}$ protein kinase was detected in the late 1980's (John et al., 1989), the relationship of its enzyme activity to cell cycle progression has not been previously reported.

In synchronous cells levels of $p34^{cdc2}$-like proteins reached a maximum level when the mitotic index peaked. Looking into the behaviour and possible identity of the four $p34^{cdc2}$-like protein bands, the levels of each changed differently during the cell cycle. These proteins are here referred to by their apparent molecular weights as indicated by electrophoretic mobility but the difference in size may be only apparent if mobility has been affected by phosphorylation. The 34 kDa protein remained relatively constant while the rest varied more than twofold. Both the 38 kDa and 36 kDa protein bands peaked in mitosis reaching 4 to 5 times as the basal level and of these two, the 38 kDa protein band was at higher level for a longer duration. The peak of the 32 kDa protein band lagged a little behind that of the 38 and 36 kDa proteins, and was closer to the time at which cells started cytokinesis. The oscillation of $p34^{cdc2}$-like protein levels during the cell cycle of *Chlamydomonas* in batch culture differs from the more constant cells detected in heterotrophic eukaryotes, such as yeast and *Xenopus* (Draetta and Beach, 1989). One possible explanation is that the oscillation in *Chlamydomonas* may have been caused by the changing growth rate in batch culture, which can induce adaptive metabolic changes that accommodate the decline in light levels per cell that occurs with increasing culture density (John et al., 1981). Enviromentally induced perturbations did not affect all proteins since levels of the non-cell cycle protein RUBISCO in the same extracts in which $p34^{cdc2}$-like protein was assayed appeared to be constant during the cell cycle (data not shown). The changes in $p34^{cdc2}$-like protein may therefore have been specific to the cell cycle. This possibility could be tested, in the

future research, by taking samples from the second cycle of a turbidostat culture in constant light, which allows environmental pressures to subside (John et al., 1981). A possible explanation for the oscillation of $p34^{cdc2}$-like protein levels is that they might be an integral part of the *Chlamydomonas* multiple fission cell division cycle. This organism has a very brief G2 phase and there is a need for its events to be recapitulated in successive round of nuclear division during multiple fission. Therefore an increasing $p34^{cdc2}$ protein level at the time of mitotic activity might be necessary to obtain with sufficient speed a level of $p34^{cdc2}$ kinase activity required for mitotic initiation in increasing numbers of nuclei. Furthermore, it is also possible that the $p34^{cdc2}$-like kinase remained constant during the cell cycle while the levels of a cdk2-like protein kinase, or other *Chlamydomonas* PSTAIR-containing kinase that is functionally similar to cdc2 or cdk2 kinase, oscillated. It has not yet been reported whether levels of cdk 2 protein kinases remain as constant as the cdc2 gene product does during the cell cycle of other eukaryotes. In *Aspergillus*, the NIMA kinase appears to act in parallel with the $p34^{cdc2}$ kinase at the G2/M transition. The level of nimA mRNA has been revealed to be under tight cell cycle regulation (Osmani, 1987, Osmani and Oakley, 1991), increasing to a maximum at mitosis and falling precipitously as the cells return to interphase (Doonan, 1992). It is possible that *Chlamydomonas* has additional cell cycle kinase, perhaps to accommodate multiple fission.

The patterns of $p34^{cdc2}$-like protein kinase activity during a cell cycle of wild-type *Chlamydomonas* has been established for the first time in the present work. Changes in the $p34^{cdc2}$-like kinase activity are similar to that in other eukaryotes and reach a maximum at mitosis. However it should be noted that the activity assayed in the present work is actually the total activity from all $p34^{cdc2}$-like protein bands since all of the four protein bands that were detected by PSTAIR antibody can bind to the $p13^{suc1}$-coupled sepharose beads during the purification of the enzyme. It is not clear how many of the four protein bands contributed to the detected enzyme activity, since at this stage it has not be possible to purified the four $p34^{cdc2}$-like protein bands separately.

The five cdc mutations studied in the present work are not necessarily directly involved in the activation or inactivation of $p34^{cdc2}$ kinase, but at least three of them do

have effects on the oscillation of the p34^{cdc2} kinase protein levels and activities during the cell cycle at restrictive temperature. The mutants *cdM-1* and *cdM-2* are somewhat similar to a temperature sensitive cell cycle mutant of *Aspergillus nidulans*, nimA5, which also arrests with doubled nuclear DNA and interphase-like cortical microtubules (Oakley and Morris, 1983). However, unlike the *nimA5* mutant that arrested with duplicated spindle pole bodies and fully activated p34^{cdc2} kinase activity (Osmani et al., 1991), the *cdM-1* and *cdM-2* mutant cells arrested with unduplicated basal bodies and only partially elevated p34^{cdc2} like protein levels and histone H1 kinase activity that correlate with early mitotic levels in normal cells. This suggests that *cdM-1* and *cdM-2* proteins are involved in the transition from late G2 into mitosis or perhaps from preprophase into mitosis.

In contrast, the *met-1* mutant, when incubated at non-permissive temperature, arrested in typical mitotic metaphase that is similar to the *bimE7* mutant of *A. nidulans* (Doonan, 1992). Arrested *met-1* cells contained a typical metaphase spindle and condensed chromosomes aligned at the metaphase plate. Both the p34^{cdc2}-like protein levels and the p34^{cdc2}-like protein kinase activity in the arrested *met-1* cells were elevated and persisted until 24 h by which time the cell cycle has normally been completed. Coincidently, a 56 kDa protein has been detected as persisting in *met-1* mutant cells using anti-p56^{cdc13} (cyclin B) antibody from fission yeast. The level of this 56 kDa protein was low in interphase cells of the mutants at both 21°C and 33°C. However, this cyclin B-like protein was abundant not only in normal mitotic cells of the *met-1* mutant that were cultured at 21°C, but also in metaphase arrested cells of the mutant that were cultured at 33°C. The high levels of this 56 kDa protein in the arrested *met-1* cells persisted in parallel with p34^{cdc2} kinase activity. Cyclin B has been identified in wide range of organisms. Although some genes that encode cyclin B like proteins have been cloned in plants (Hata et al., 1991; Hirt et al, 1992) and levels of cyclin B like mRNA have also been investigated in higher plant cells (Hirt et al., 1992), the tight coupling of a cyclin B-like protein with p34^{cdc2}-like activity observed here is the first evidence of this kind from the plant kingdom.

The $p13^{suc1}$-like binding protein of $p34^{cdc2}$ has also been identified in *Chlamydomonas* (John et al., 1991). In the present study, analysis of the $p13^{suc1}$-like protein in a G2-arresting mutant *cdM-1* and the metaphase arresting mutant *met-1* revealed that levels of this $p34^{cdc2}$ binding protein were constant both during the normal cell cycle of the two mutant cells that were cultured at permissive temperature and in the arrested mutant cells that were cultured at restrictive temperature. Therefore, although $p13^{suc1}$ is necessary for completion of mitosis (Moreno et al., 1989a) there is no evidence that abnormal $p13^{suc1}$ levels are the cause of arrest in *cdM-1*, *cdM-2* or *met-1*.

Coordination of mitosis and cytokinesis is important for successful division and may be regulated by "checkpoint" controls that normally only allow inactivation of $p34^{cdc2}$ kinase when a fully functional spindle and metaphase array of chromosomes have been established. In fission yeast, a gene product identified by a *cdc16*$^{-}$ mutant has a function of "checking" for presence of a mitotic spindle and allowing the inactivation of $p34^{cdc2}$ kinase only if a spindle has been formed (Fankhauser et al., 1993; Chang and Nurse, 1993). According to the current hypothesis establishment of a spindle allows a change of state of the *cdc16* gene product that allows the cdc2 kinase to be inactivated and therefore anaphase to proceed (Chang and Nurse, 1993). A functional homologue of *cdc16* gene in budding yeast is the mitotic checkpoint gene *BUB2* which shares 39% identity with the *cdc16* gene and can complement the *cdc16*st fission yeast mutant, indicating that this sort of check point control may be conserved in many organisms (Chang and Nurse, 1993).

In relation to $p34^{cdc2}$ kinase in *Chlamydomonas*, arrested cells were observed to have either partially elevated and persisting $p34^{cdc2}$-like protein and enzyme activity, as in the case of *cdM-1* and *cdM-2*; or fully elevated $p34^{cdc2}$-like protein and enzyme activity, as in *met-1*; or in wild type cells treated with oryzalin an apparently normal elevation and decline of $p34^{cdc2}$-like protein and activity could occur. The possible relevance of check point mechanism to these patterns can be considered.

The antimicrotubule drug oryzalin has parallels with benomyl that acts against fungal tubulins and nocodazole that acts against animal tubulins. However, different microtubule depolymerising drugs seem to have different effects in diverse organisms.

The microtubule depolymerising drug nocodazole arrests HeLa cells in metaphase with elevated $p34^{cdc2}$ kinase activity (Draetta and Beach, 1988), and benomyl also prevented budding yeast cells forming normal spindles and resulted in high $p34^{cdc2}$ kinase activity (Hoyt et al. 1991), whereas transient activation of $p34^{cdc2}$ kinase has seen in *Xenopus* oocytes treated with antimicrotubule drug (Hara et al., 1980; Murray, 1989) and no activation at all was seen in fission yeast treated with the antimicrotubule drug thiabendazole that resulted in a block to both dephosphorylation and activation of $p34^{cdc2}$ (Alfa et al., 1990).

Inactivation of checkpoint genes in budding yeast has shown that there is no structural reason why a nuclear envelope cannot form around chromosomes that have failed to separate at mitosis because antimicrotubule agents have prevented spindle formation. Rather the nuclear envelope does not normally reform because a checkpoint only allows it to occur after completion of a spindle. When the checkpoint genes are inactivated by mutation then completion of mitosis is attempted even with an abnormal or absent spindle. The genes identified by Hoyt et al (1991), *BUB1*, *BUB2* and *BUB3*, caused death in cells that attempted to complete mitosis in the presence of high concentrations of benomyl while wild type cells survived because they arrested in mitosis under these circumstances until normal spindle function was completed. Similarly the genes identified by Li and Murray (1991), *MAD1*, *MAD2* and *MAD3*, also caused death in cells dividing in the presence of low concentration of benomyl which slowed spindle function. Normal cells progressed proportionately slower through mitosis but the mutants did not slow and they died. *Chlamydomonas* cells also seem to have a checkpoint for spindle function because cells treated until 16 h with oryzalin delayed mitosis but completed it rapidly on removal of the inhibitor.

The mechanism of normal arrest following disruption of spindle function includes a high and persisting $p34^{cdc2}$ kinase activity (Hoyt et al., 1991; Li and Murray, 1991) which is incompatible with progress through anaphase (Murray et al., 1989; Ghiara et al., 1991). In the *BUB* and *MAD* mutants of budding yeast, and in the *cdc16* mutant of fission yeast (Frauhauser et al., 1993), high levels of $p34^{cdc2}$ kinase activity are not

maintained when the spindle is blocked and this may be the reason that the checkpoint is unable to arrest progress.

The check on the spindle at the metaphase anaphase transition probably only detects some aspect of spindle formation (Chang and Nurse, 1993) such as duplication and separation of the spindle poles. Consistent with this possibility, oryzalin treated wild type *Chlamydomonas* cells, which had depolymerised cortical and spindle microtubules but maintained intact basal bodies (stabilised by acetylation), maintained a normal oscillation of the $p34^{cdc2}$ kinase activity (Fig. 3.3.18) perhaps because the duplicated and separated basal bodies satisfied a spindle check.

Checkpoint controls are not of obvious relevance to the arrest behaviour of the G2 mutants *cdM-1* and *cdM-2*. These mutants do suggest that an acceleration of $p34^{cdc2}$ activation is an essential part of fully initiating prophase since the mutants at restrictive temperature were unable to exceed a partial activation (Fig. 3.3.7 and Fig. 3.3.8) and were also unable to enter prophase.

However, arrest of the *met-1* mutant could be explained by a checkpoint control or its failure. First, a checkpoint could correctly detect that the cells have not fully attained metaphase although no defect is visible. Second, the possible checkpoint mechanism itself could be altered by the mutation and incorrectly respond to a perfect metaphase as if it is aberrant. Alternatively, the signal to inactivate $p34^{cdc2}$ that normally follows metaphase may be imperfectly given. Finally, the $p34^{cdc2}$ kinase inactivation mechanism maybe unable to respond to the signal that initiates anaphase. Such a failure of inactivation could arise if the *met-1* mutation alters a protein equivalent to an E3-type ubiquitinisation protein that normally catalyses attachment of ubiquitin to cyclin at metaphase prior to the degradation of the cyclin and consequent inactivation of the kinase (Murray and Kirschner, 1989; reviewed by Vierstra , 1993). It is consistent with this last possibility that a cyclin-like protein was seen to persist in arrested *met-1* cells, however this persistence is also consistent with the three earlier-listed possibilities.

From this discussion of the diversity of possible biochemical lesions in the *met-1* mutant it is apparent that full understanding of the function of this gene, and the other cell cycle genes studied here, may require cloning of these genes. However,

Chlamydomonas falls far short of yeast as a cloning vehicle (Holmes and Dutcher, 1993) because it lacks a cloning vector containing an effective autonomous replication sequence (ARS) for the plant cell, and because techniques giving high transformation rates have not yet been developed. However, *Chlamydomonas* transformation has recently been obtained using a glass bead method for partial cell fracture in the presence of external DNA (Kindle et al., 1992) and ARS sequences are being sought in several laboratories (Kindle et al., 1992; Purton et al., 1992, Personal communication).

In the present study, preliminary attempts have been made to clone the *met-1* gene. A first attempt was by mapping the *met-1* mutation to a particular genetic linkage group. If a closely linked marker had previously been cloned, or if the mapping location corresponded with a cloned RFLP marker, then the cloned DNA would have been useful as a probe for isolating the *met-1* gene from a library. By crossing the *met-1* mutant with different cell lines that carried known genetic markers for each linkage group the *met-1* mutation was mapped to linkage group XIV. The *met-1* gene is therefore the first cell division cycle gene that has been mapped in *Chlamydomonas* and has resulted in an extension of linkage group XIV. Unfortunately, *met-1* has been found in a linkage group with few markers and no cloned genes. However, the exercise of mapping of *met-1* itself is significant as a part of the characterisation of the mutant.

Since no closely linked gene is available as a probe an alternative means of cloning by complementation has been considered. For this purpose a total genomic DNA library has been constructed (data not shown in the thesis). The glass bead method for transformation (Kindle, 1992; Purton et al., 1992') was used to complement an arginine requiring mutant with a cloned arginine biosynthetic gene and the observed transformation rate of $3x10^{-6}/\mu g$ DNA indicated that the method might be used in the future for *met-1* transformation with a genomic DNA library to complement the *met-1* mutant. With present resources the complemented gene cannot be simply recovered. It would be necessary to construct a second genomic library from the complemented clone and probe with vector sequences. The same strategy could also be used for other *Chlamydomonas* cdc mutants. Although study of the cell cycle control using *Chlamydomonas* as a model system is still in its infancy, the advantages of this unicellular

plant for genetic and biochemical analysis of the cell cycle encourage the view that it provides a useful resource for study of plant cell division.

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